“Never let anyone tell you that you can’t do something,
keep moving fearlessly toward the horizon of your dreams”.

UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Intersectoral Innovation

(Class XXXVIII)

Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy

Doctoral thesis

DEVELOPMENT OF NOVEL BIOTECHNOLOGICAL APPROACHES FOR ACCELERATING GENETIC GAIN IN THE BOVINE

VET-01

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Summary

Modern livestock production is increasingly challenged to improve reproductive efficiency while maintaining sustainability and animal welfare standards. Accelerating genetic gain in cattle (*Bos taurus*) is essential for breeding animals that are more resilient to climate change, more resistant to disease, and capable of higher meat and milk productivity—all while minimizing their environmental footprint. One possible strategy to achieve this goal is to significantly shorten the generation interval by producing gametes directly from embryos.

Current evidence indicates that a fully developed offspring can be obtained by injecting a spermatid into a fully matured oocyte. In contrast, fertilization cannot be achieved by injecting a mature sperm into an immature oocyte. Based on this, the focus of this work was placed on the generation of male haploid cells in bovine species.

However, *in vitro* spermatogenesis remains a major challenge in large mammals and has, to date, been successfully accomplished only in rodents. To address this limitation, a stepwise approach was adopted to develop targeted techniques for some of the phases of the process required to recapitulate the differentiation of embryonic cells into the

spermatogonial lineage and to subsequently support their progression through complete meiosis.

The initial experiments aimed to establish a protocol for isolating immature bovine spermatids and evaluating their fertilization potential using micromanipulation techniques. This was followed by the optimization of a decellularization protocol to create a testicular biological scaffold that mimics the native tissue microenvironment, preserving its structural, biomechanical, and biochemical properties. This scaffold was designed to support *in vitro* meiosis. The final set of experiments focused on isolating and characterizing key functional testicular cell types—Sertoli cells and spermatogonia—which are essential for meiotic progression and intended to repopulate the scaffold.

The results demonstrated successful isolation of bovine spermatids, with significant enrichment of haploid subpopulations. These cells exhibited high haploidy rates, expressed spermatid-specific transcripts (PRM1, PRM2, SPACA9, SPERT), and showed cytoplasmic localization of SPERT protein. DNA integrity was maintained after 24 hours at both 4°C and 37°C, although mitochondrial activity and reactive oxygen species (ROS) levels increased over time. Despite these promising features, spermatids showed limited fertilization capacity following intracytoplasmic injection into *in vitro* matured oocytes.

The second phase of the study established an efficient decellularization protocol for bovine testicular tissue, achieving thorough removal of cellular components while preserving the extracellular matrix architecture. A 12-hour exposure to SDS provided the optimal balance between decellularization and matrix integrity. These scaffolds supported fibroblast repopulation, confirming their biocompatibility.

In the final part of the thesis, fibroblasts, Sertoli cells, and spermatogonia were successfully isolated and characterized using specific markers—vimentin, SOX9, and PGP9.5, respectively. These cell populations represent the essential building blocks for developing a functional 3D *in vitro* model of the testicular microenvironment, which may support spermatogonial differentiation and, potentially, completion of the meiotic process.

Overall, this work established protocols for a few key steps toward the ultimate goal of generating male haploid cells suitable for injection into mature oocytes. Although the task confirmed to be highly complex, the gradual and systematic approach adopted in this study contributes to laying the groundwork for future advancements in the field.

CHAPTER 1:
Introduction

Sustainable and resilient livestock farming

Livestock farming is at the core of global food systems, providing essential proteins, livelihoods, and economic opportunities (FAO, 2023). However, the sector faces several pressing challenges, including the accelerating impact of climate change, increasing global food demand driven by population growth, the need to combat infectious diseases, and the imperative to reduce environmental impact, particularly methane emissions (Kyriazakis et al., 2024).

These challenges are deeply interconnected. For instance, global temperatures are projected to rise by approximately 2.7°C by 2100 (Grant et al., 2025) (Figure 1), a change that will have profound consequences for ecosystems, agriculture, and both human and animal health.

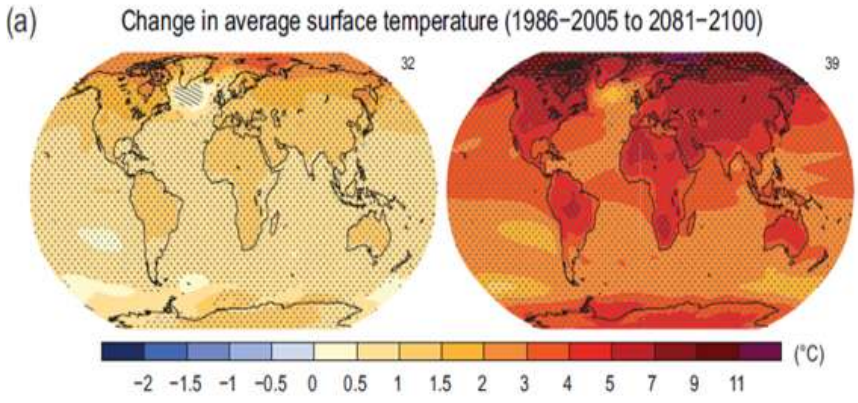

Figure 1. Projected global average temperature increases under different greenhouse gas emission scenarios (SSPs) up to the year 2100. The European Environment - State and outlook **2015** EN PDF: TH-01-15-001-EN-C-ISBN: 978-92-9213-515-7 - doi:10.2800/944899

Rising temperatures are expected to exacerbate water scarcity, reduce agricultural productivity, and increase the prevalence of heat stress-related illnesses (Godde et al., 2021), further intensifying the pressures on livestock systems. Within animal production, heat stress can impair reproduction, reduce feed intake, and compromise overall animal welfare (Nardone et al., 2010; Sejian et al., 2018). As global average temperatures continue to rise, understanding and mitigating the impacts of heat stress is becoming increasingly critical for ensuring sustainable development and food security (Hong et al., 2025; Saleem et al., 2025).

Global warming has intensified environmental challenges, particularly in livestock production systems, which are increasingly affected by heatwaves, droughts, and unpredictable resource availability (Bashiru & Oseni, 2025). From a reproductive standpoint, heat stress disrupts hormonal balance in both male and female animals, ultimately reducing fertility (Țogoe & Mincă, 2024). In females, it impairs follicular development, lowers oocyte quality, induces silent estrus, and increases the risk of pregnancy loss (Khan et al., 2023). In males, heat stress compromises sperm quality, including motility and morphology, increases oxidative stress, impairs spermatogenesis, and reduces libido (Walke et al., 2023).

Beyond elevated temperatures, altered precipitation patterns affect forage and water availability, indirectly compromising nutritional status and reproductive performance (Rust, 2018). Climate change also influences the distribution and prevalence of pathogens and vectors (e.g., insects), increasing the risk of infectious diseases and further challenging reproductive health (Özkan et al., 2016). Indeed, heat stress significantly impairs the immune system of cattle, reducing the activity of lymphocytes, neutrophils, and macrophages, key components of both innate and adaptive immunity (Bagath et al., 2019). This weakened immune response leads to a higher incidence and severity of infections. Respiratory conditions such as pneumonia, bronchitis, and bovine respiratory disease complex are particularly common in heat-stressed cattle due to impaired respiratory function and increased susceptibility to pathogens (Bagath et al., 2019). Additionally, heat stress raises the incidence of mastitis, a costly inflammation of the mammary gland, as the compromised immune system becomes less effective in countering bacterial infections (Lengi et al., 2022). Other infections, including enteric diseases (Bett et al., 2017) and metritis (Molinari et al., 2022), are also exacerbated under heat stress conditions.

The combined impacts of heat stress and disease pose serious threats to animal welfare, productivity, and the economic stability of the livestock

sector. Reduced milk production, slower growth rates, and higher mortality due to heat-induced illnesses contribute to significant financial losses (Wankar et al., 2021).

Another critical concern is the contribution of livestock to greenhouse gas (GHG) emissions and air pollution. Livestock production is a major source of GHGs, with substantial emissions arising from manure management, including housing, storage, treatment, and land application. The main gases emitted are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), all of which are potent contributors to climate change(Asem-Hiablie et al., 2019). Among these, methane is particularly concerning due to its significantly higher global warming potential compared to CO_2 (Emmerling et al., 2020). Reducing methane emissions is therefore a critical priority. Mitigation strategies such as chemical inhibitors (e.g., 3-nitrooxypropanol or 3-NOP), dietary lipid supplementation, and improved feed efficiency have shown promise in lowering emissions without compromising productivity (Z. Liu et al., 2023). As global temperatures rise and climate variability intensifies, it is imperative to develop more resilient animals that can better tolerate heat stress, resource limitations, and emerging diseases (Bernabucci et al., 2014). At the same time, the global population is expected to reach 9.7 billion by 2050 (Figure 2),

further amplifying the demand for sustainable, high-quality animal protein (FAO, 2017).

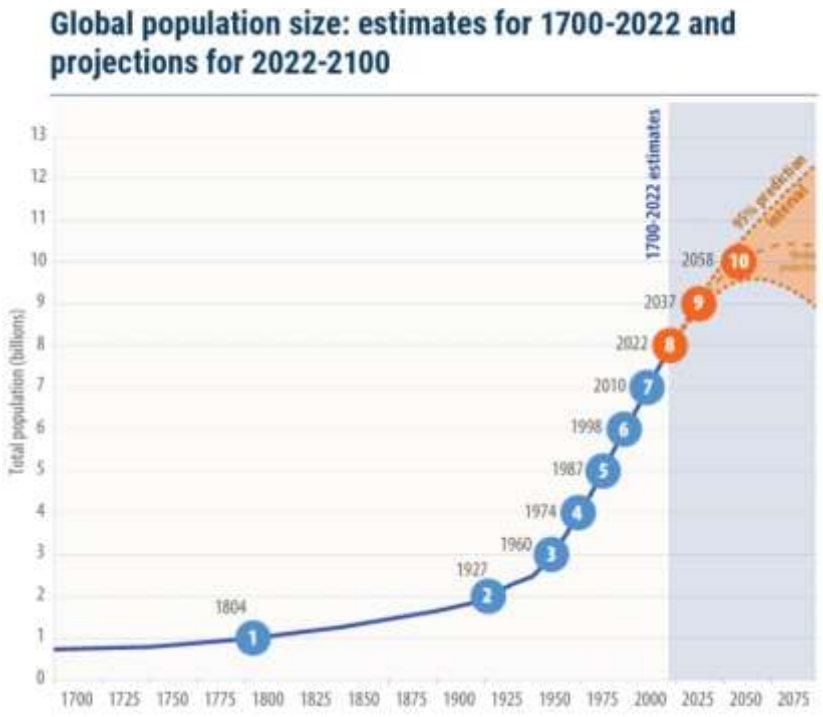

Figure 2. The global population is expected to exceed 9 billion by around 2037 and reach 10 billion by approximately 2058. Zeifman, L., Hertog, S., Kantorova, V., & Wilmoth, J. (2022). A world of 8 billion.

As the demand for animal products continues to grow, improving productive performance through genetic and technological approaches has become a priority for both research and industry (Eastwood et al., 2021). Sustainable and resilient livestock farming focuses on minimizing environmental impact, improving animal welfare, and ensuring economic viability, while increasing the capacity of production systems to cope with climate-related and the other related challenges. This approach requires

the integration of environmental, social, and economic factors within livestock management practices.

Genetic gain, selection criteria and ART

Genomic selection (GS) has revolutionized cattle breeding by enabling the identification and propagation of superior genetic traits with unprecedented precision and speed (García-Ruiz et al., 2016; Hayes et al., 2009; Meuwissen et al., 2001; Van Eenennaam & Young, 2014). It has proven particularly effective in enhancing meat quality traits such as tenderness and water-holding capacity (Hocquette et al., 2007; Kasimanickam et al., 2025). These advancements have been supported by the integration of genome-wide association studies (GWAS), marker-assisted selection (MAS), and emerging genome-editing technologies like CRISPR/Cas9 (Raj et al., 2025). The scope of GS has expanded beyond production traits to include disease resistance and reproductive (Arya et al., 2024) Multi-omics approaches—integrating genomics, transcriptomics, proteomics, and metabolomics—can be combined with GS to gain deeper insights into complex traits (Wadood et al., 2025). Recent advances in gene editing have further enhanced GS by enabling direct genetic modifications aimed at improving animal productivity and

resilience (Van Eenennaam, 2019). Additionally, incorporating traits related to animal health and welfare, such as robustness, longevity, and disease resistance into breeding objectives is becoming increasingly important (Friggens et al., 2017; Rauw & Gomez-Raya, 2015). Achieving sustainable genetic gain in cattle requires a comprehensive strategy that integrates genomic innovation (Van Eenennaam, 2025), reproductive efficiency (Menchaca et al., 2018), environmental adaptability (Naskar et al., 2012), and ethical responsibility (Sultan et al., 2023).

When combined with reproductive technologies such as artificial insemination (AI), embryo transfer (ET), and *in vitro* fertilization (IVF), the impact of genomic selection is significantly amplified—particularly in breeding programs focused on shortening generation intervals and increasing selection efficiency (Baruselli et al., 2023).

A wide range of assisted reproductive technologies (ARTs) are currently employed to enhance livestock production efficiency. These include cryopreservation of sperm and embryos, estrus synchronization, multiple ovulation and embryo transfer (MOET), ovum pick-up (OPU), *in vitro* embryo production (IVP), sex determination of sperm or embryos, and nuclear transfer (NT) (Mueller & Van Eenennaam, 2022).

Currently, the most widely used techniques globally are AI and timed artificial insemination (TAI), which significantly enhance reproductive

and productive efficiency by enabling the insemination of anestrous cows with GS (Lamb & Mercadante, 2016). While these innovative procedures offer substantial improvements, their potential is maximized when combined with genomic selection (GS) and genomic editing (GnEd). These technologies present promising opportunities to introduce beneficial genetic variation across cattle breeds without undesired linkage drag and even incorporate advantageous traits from other species(Frattini et al., 2017). GnEd experiments in cattle have primarily focused on three key areas: (1) animal health and welfare, (2) product yield and quality, and (3) reproduction and novel breeding strategies (Mueller & Van Eenennaam, 2022). Despite its potential, GnEd has not yet been widely adopted commercially due to its complexity, particularly the requirement for homozygous modifications in many applications (Bishop & Van Eenennaam, 2020). The integration of GS and ART represents a powerful strategy for accurately selecting genetically superior young animals, thereby substantially reducing generation intervals and accelerating genetic gain (Mikkola et al., 2024).

Shortening the Generation Interval in Bovine Species

Despite all the advances described above, genetic progress is constrained by the **generation interval**—the average age of parents when their

offspring are born. This parameter is a major biological bottleneck in accelerating genetic gain (Bijma & Woolliams, 1999). Shortening the generation interval would enable faster turnover of generations and more frequent incorporation of superior genetics into the population. The rate of genetic progress can be expressed by the breeder's equation:

$$\Delta G = \frac{i \cdot r \cdot \sigma_A}{L}$$

Where:

- ΔG *= genetic gain per year*
- i *= selection intensity*
- r *= accuracy of selection*
- σ_A *= additive genetic standard deviation*
- L *= generation interval (in years)*

Reducing the generation interval (L) significantly increases annual genetic gain (ΔG), assuming other factors remain constant (Decker, 2021). By decreasing the time between generations, favorable alleles spread more rapidly, and the number of selection cycles within a given timeframe rises (Kasimanickam et al., 2025). This is particularly relevant in cattle, where long reproductive cycles and delayed sexual maturity naturally slow genetic improvement (Berglund, 2008).

One promising strategy involves deriving gametes from embryos that have been genetically screened at an early stage (Figure 3).

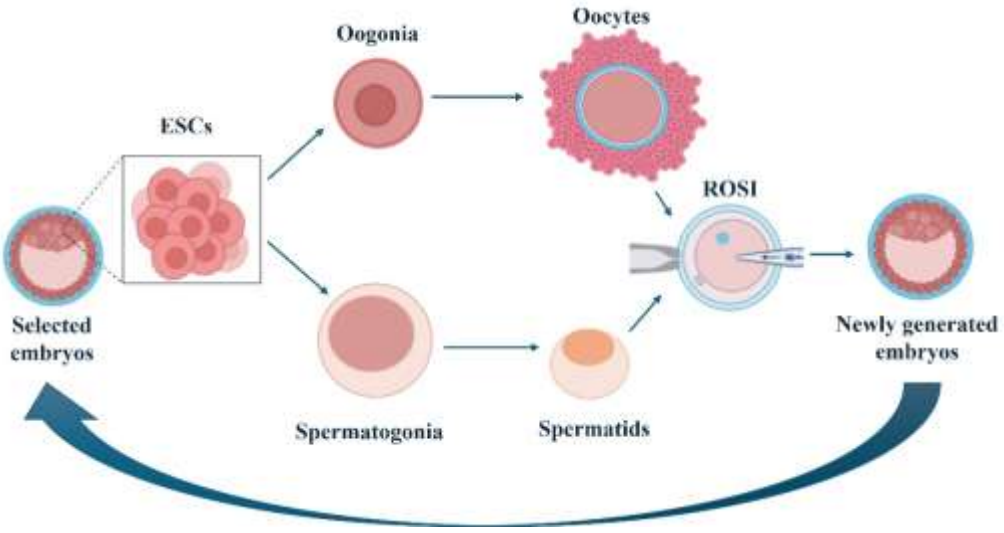

Figure 3. Schematic representation of gamete derivation from embryos through advanced reproductive technologies.

This approach would allow the use of genetically superior individuals at the embryonic stage thereby significantly accelerating the dissemination of desirable alleles. Male embryos would be especially advantageous because *in vitro* spermatogenesis appears more feasible and a single male can sire numerous offspring (Wang et al., 2023).

Biologically, spermatogenesis is a continuous and externally accessible process throughout the male's reproductive life, making it more amenable to *in vitro* modeling (Kulibin & Malolina, 2023). In contrast, oogenesis is highly regulated, temporally restricted, and involves prolonged meiotic arrest and complex somatic interactions (Li & Albertini, 2013). Moreover,

oocytes play critical roles beyond fertilization, including supporting early embryonic development, providing maternal transcripts and organelles, and ensuring proper epigenetic reprogramming (Chen & Qiu, 2012; Martin et al., 2016; Reader et al., 2017). These requirements make *in vitro* oocyte maturation significantly more challenging.

Conversely, sperm cells, though requiring precise chromatin remodeling and flagellar development, do not bear the burden of sustaining embryogenesis, simplifying functional validation. Current research showed that the induction of spermatogonial differentiation and meiosis *in vitro* has shown promising results, particularly in rodents (Matsumura et al., 2021; Sato et al., 2013). Translating these findings to bovine systems remains challenging, but the relative biological simplicity of male gametes offers a practical starting point for developing effective *in vitro* protocols.

Comparative overview of bovine and mouse male reproductive systems

The mammalian testis exhibits considerable interspecies variation in size, architecture, and spermatogenic dynamics, which critically influences the feasibility of *in vitro* spermatogenesis. In cattle (*Bos taurus*), testes are large and vertically oriented, measuring 10–15 cm in length and weighing 200–500 g. They contain a complex network of seminiferous tubules and

a heterogeneous cellular composition (Staub & Johnson, 2018). In contrast, the mouse (*Mus musculus*) testis is compact, approximately 1 cm long and 130–160 mg in weight, highly vascularized and composed of tightly packed seminiferous tubules with a simplified and well-characterized cellular architecture (Yoshida, 2020) (Figure 4).

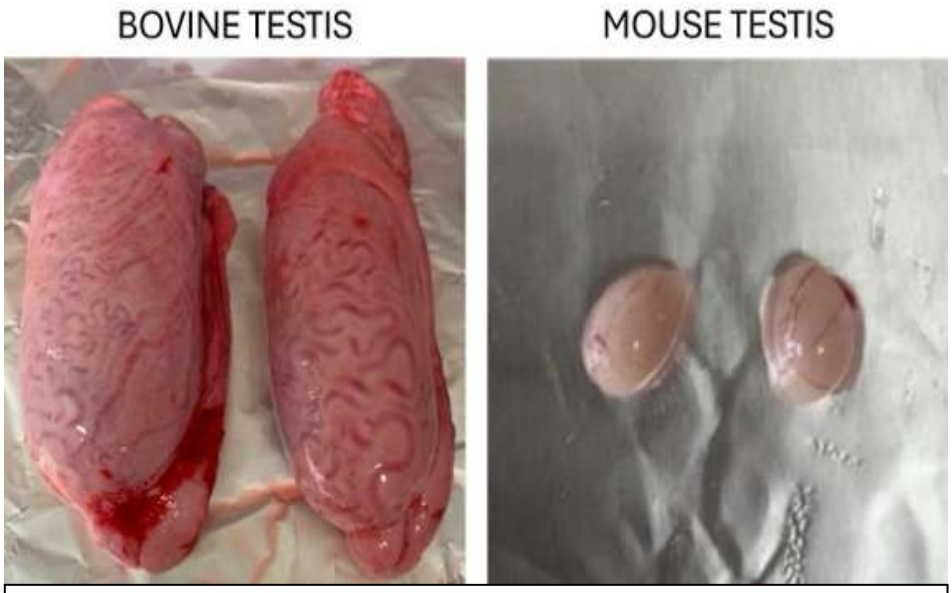

FIGURE 4. Macroscopic comparison between bovine and mouse testes. The marked difference in size and morphology reflects species-specific reproductive physiology and testicular organization.

Spermatogenesis in both species follows a conserved sequence: mitotic proliferation of spermatogonia, meiotic division of spermatocytes, and spermiogenesis—the morphological transformation of spermatids into spermatozoa. However, the organization and regulation of these phases differ markedly. In mice, the entire spermatogenic process takes

approximately 35–38 days, with a seminiferous epithelium cycle of 8.6–8.8 days (Griswold, 2016). In bulls, spermatogenesis is significantly longer, lasting around 61 days, with a seminiferous epithelium cycle of 13.5 days (Staub & Johnson, 2018).

Mouse spermatogenesis is supported by a flexible stem cell system, with undifferentiated spermatogonia residing in a vasculature-associated niche that facilitates continuous sperm production and efficient nutrient diffusion (Yoshida, 2008; Yoshida et al., 2007). This anatomical and physiological simplicity has enabled successful *in vitro* spermatogenesis using organ culture (Shuchat et al., 2022) and microfluidic technologies (Komeya et al., 2016). Conversely, bovine spermatogenesis is more intricate, rigid spatial organization of germ and somatic cells, and higher metabolic demands (Barth et al., 2025; Staub & Johnson, 2018). The bovine seminiferous epithelium presents a complex wave-like arrangement of cell associations, and spermatogonial renewal involves multiple mitotic divisions with less clearly defined stem cell niches (Barth et al., 2025; Staub & Johnson, 2018).

These complexities (Table 1) hinder replication of the bovine testicular microenvironment *in vitro*, particularly in supporting complete meiotic progression. While mouse models have provided extensive insights into

germ cell differentiation and meiotic regulation (Ishiguro, 2023), translating these findings to bovine systems remains challenging. Bovine germ cells often arrest at early meiotic stages *in vitro* (Kulibin & Malolina, 2023), and the generation of functional haploid cells has not yet been reliably achieved. This underscores the need for species-specific strategies

Feature	Mouse testis	Bovine testis
Size	~1cm long;~0.13-0.16 g	10-15 cm long, 200-500 g
Location	Abdominal/Inguinal	Scrotal, anterior prepubic region
Sperm output	Low (sufficient for small litters)	Very high (support large-scale reproduction)
Seminiferous tubules	Shorter, less convoluted	long, higly convoluted, dense
Leyding cells	Present, moderate testosterone production	Abudant, high testosterone output
Vascularization	Simple	Extensive, withpampiniform plexus for thermoregulation
Thermoregulation	Minimal (internal testis)	Complex (scrotal location, vascular heat exchange)
Maturation time	~6-8 weeks	~6-12 months
Spermatogenesis cycle	~35 days	~60 days

Table 1. *The table summarizes the main differences between bovine and mouse testes, highlighting species-specific variations in size, anatomy, and seminiferous tubule organization.*

that account for the unique structural and regulatory features of large mammalian testes in developing effective *in vitro* spermatogenesis protocols.

The anatomical differences between cattle and mice, particularly in reproductive physiology and gametogenesis, present distinct challenges trasitioning from one species to the other.

Building upon the comparative anatomical insights between bovine and murine testes, the review below (Pasquariello et al., 2024) explores how reproductive technologies can be strategically employed to accelerate the generational turnover in ruminants, thereby enhancing the efficiency of genetic improvement programs.

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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

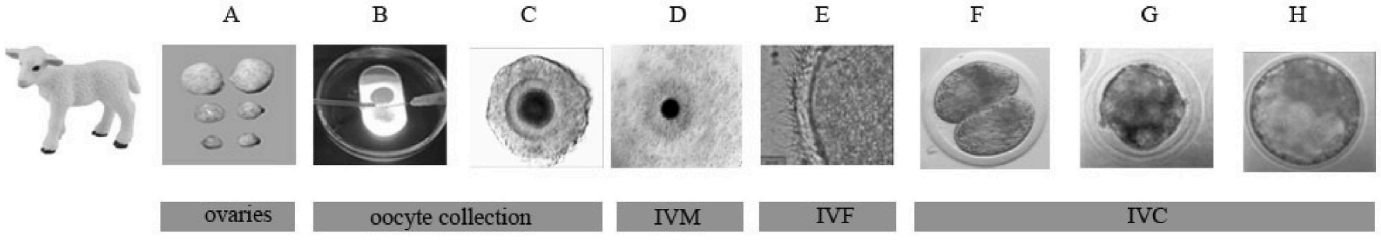

Fig. 1. Schematic sequence of steps in IVEP from prepubertal ewes (30–40 days old): ovaries with different morphology obtained from slaughtered lambs (A), ovarian slicing for oocytes collection (B); *in vitro* maturation (IVM) of oocytes cultured for 24 h: cumulus-oocyte complex (COC) (C), cumulus expansion 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

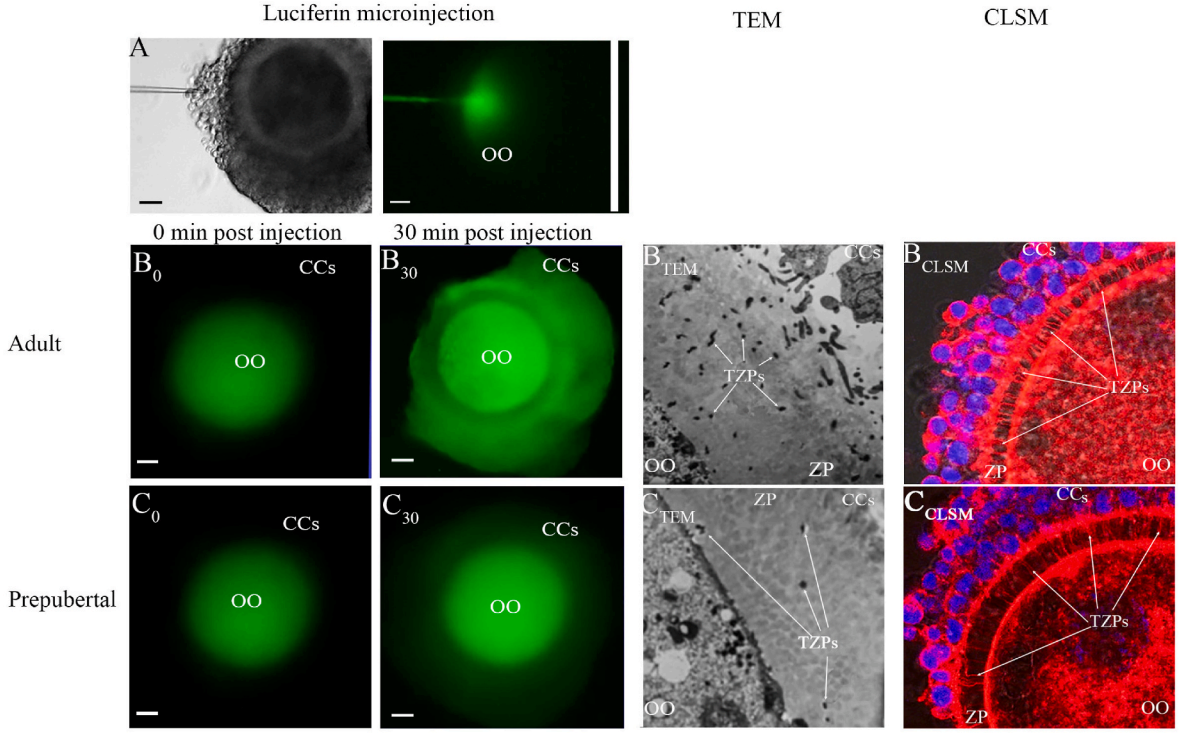

Fig. 2. Functional and structural intercellular communications in the cumulus–oocyte complexes (COCs) of adult and prepubertal ovine oocytes. Injection of the fluorescent dye Lucifer Yellow (LY) into oocyte cytoplasm by a microneedle (A). Junctional diffusion of LY from oocyte to cumulus cells (CCs): 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

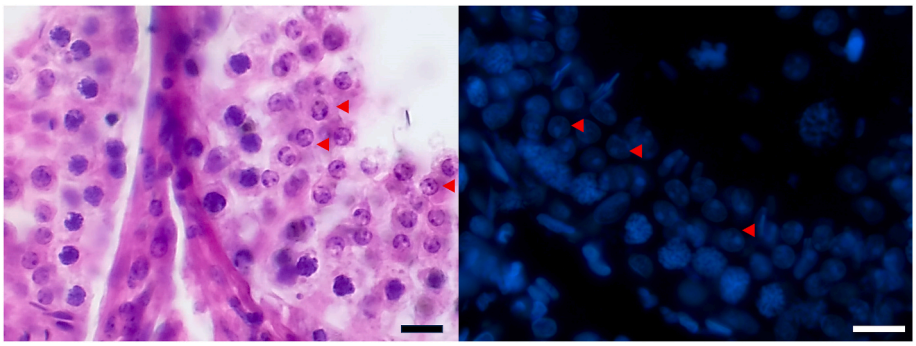

Fig. 3. Representative pictures showing cell and nuclear morphology of round spermatids (indicated with red arrows) in the seminiferous tubule of post-puberal bovine testis. The images were produced using 5 μm-thick histological sections of formalin fixed, paraffin embedded testicular tissues which were 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

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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.

References

[1] Ritchie H, Roser M. Yields vs. land use: how the Green Revolution enabled us to feed a growing population. Our World in Data 2024;33:737.
[2] Henchion M, Moloney AP, Hyland J, Zimmermann J, McCarthy S. Review: trends for meat, milk and egg consumption for the next decades and the role played by livestock systems in the global production of proteins. Animal 2021;15(Suppl 1):100287.
[3] Berglund B. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect Ecol Epidemiol 2015;5:28564.
[4] Amann RP, DeJarnette JM. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: a paradigm shift. Theriogenology 2012;77:795–817.
[5] VanRaden PM. Efficient methods to compute genomic predictions. J Dairy Sci 2008;91:4414–23.
[6] VanRaden PM, Cooper TA. Genomic evaluations and breed composition for crossbred U.S. dairy cattle. Interbull Bull. 2015;49:19–23.
[7] Aguilar I, Misztal I, Johnson DL, Legarra A, Tsuruta S, Lawlor TJ. Hot topic: a unified approach to utilize phenotypic, full pedigree, and genomic information for genetic evaluation of Holstein final score. J Dairy Sci 2010;93:743–52.
[8] Schaeffer LR. Strategy for applying genome-wide selection in dairy cattle. J Anim Breed Genet 2006;123:218–23.
[9] Cenariu M, Pall E, Cernea C, Groza I. Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability. J Biomed Biotechnol 2012;2012:541384.
[10] Lafontaine S, Labrecque R, Blondin P, Cue RI, Sirard MA. Comparison of cattle derived from in vitro fertilization, multiple ovulation embryo transfer, and artificial insemination for milk production and fertility traits. J Dairy Sci 2023;106:4380–96.
[11] Campolina Diniz Peixoto MG, Carrara ER, Lopes PS, Tomita Bruneli FA, Penna VM. The contribution of a MOET nucleus scheme for the improvement of Guzera (Bos indicus) cattle for milk traits in Brazil. Front Genet 2022;13:982858.
[12] McDaniel BT, Cassell BG. Effects of embryo transfer on genetic change in dairy cattle. J Dairy Sci 1981;64:2484–92.
[13] Sirard MA. 40 years of bovine IVF in the new genomic selection context. Reproduction 2018;156:R1–7.
[14] van Wagtendonk-de Leeuw AM. Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. Theriogenology 2006;65:914–25.
[15] Goszczynski DE, Cheng H, Demyda-Peyrás S, Medrano JF, Wu J, Ross PJ. In vitro breeding: application of embryonic stem cells to animal production. Biol Reprod 2018;100:885–95.
[16] Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science 2012;338:971–5.
[17] Hayashi K, Saitou M. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. Nat Protoc 2013;8:1513–24.
[18] Armstrong DT, Holm P, Irvine B, Petersen BA, Stubbings RB, McLean D, et al. Pregnancies and live birth from in vitro fertilization of calf oocytes collected by laparoscopic follicular aspiration. Theriogenology 1992;38:667–78.
[19] Armstrong D, Irvine B, Earl C. In vitro fertilisation of follicular oocytes from juvenile lambs and their developmental competence in vitro and in vivo. Biol Reprod 1994;50(Suppl):189.
[20] Earl CRA, Irvine B, Armstrong D. Development of techniques for the production of viable embryos from 6 to 7 week old lambs. Proc Aust Soc Anim Prod 1994;20:428.
[21] Izquierdo D, Catalá MG, Paramio MT. Small ruminants: prepubertal oocyte donors. Methods Mol Biol 2019;2006:155–63.
[22] Currin L, Michalovic L, Bellefleur A-M, Gutierrez K, Glanzner W, Schuermann Y, et al. The effect of age and length of gonadotropin stimulation on the in vitro embryo development of Holstein calf oocytes. Theriogenology 2017;104:87–93.
[23] Baldassarre H, Wang B, Kafidi N, Keefer C, Lazaris A, Karatzas CN. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and in vitro embryo production technologies. Theriogenology 2002;57:275–84.
[24] Morton KM, Catt SL, Maxwell WMC, Evans G. Effects of lamb age, hormone stimulation and response to hormone stimulation on the yield and in vitro developmental competence of prepubertal lamb oocytes. Reprod Fertil Dev 2005;17:593–601.
[25] Leoni GG, Palmerini MG, Satta V, Succu S, Pasciu V, Zinellu A, et al. Differences in the kinetic of the first meiotic division and in active mitochondrial distribution between prepubertal and adult oocytes mirror differences in their developmental competence in a sheep model. PLoS One 2015;10:e0124911.
[26] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, et al. In vitro production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reprod Fertil Dev 2009;21:901–8.
[27] O'Brien JK, Beck NF, Maxwell WM, Evans G. Effect of hormone pre-treatment of prepubertal sheep on the production and developmental capacity of oocytes in vitro and in vivo. Reprod Fertil Dev 1997;9:625–31.
[28] Revel F, Mermillod P, Peynot N, Renard JP, Heyman Y. Low developmental capacity of in vitro matured and fertilized oocytes from calves compared with that of cows. J Reprod Fertil 1995;103:115–20.
[29] Armstrong DT, Kotaras PJ, Earl CR. Advances in production of embryos in vitro from juvenile and prepubertal oocytes from the calf and lamb. Reprod Fertil Dev 1997;9:333–40.
[30] Baruselli PS, Rodrigues CA, Ferreira RM, Sales JNS, Elliff FM, Silva LG, et al. Impact of oocyte donor age and breed on in vitro embryo production in cattle, and relationship of dairy and beef embryo recipients on pregnancy and the subsequent performance of offspring: a review. Reprod Fertil Dev 2021;34:36–51.
[31] Currin L, Michalovic L, Bellefleur AM, Gutierrez K, Glanzner W, Schuermann Y, et al. The effect of age and length of gonadotropin stimulation on the in vitro embryo development of Holstein calf oocytes. Theriogenology 2017;104:87–93.
[32] Ledda S, Bogliolo L, Leoni G, Naitana S. Production and lambing rate of blastocysts derived from in vitro matured oocytes after gonadotropin treatment of prepubertal ewes. J Anim Sci 1999;77:2234–9.
[33] Morton KM, Catt SL, Maxwell WM, Evans G. An efficient method of ovarian stimulation and in vitro embryo production from prepubertal lambs. Reprod Fertil Dev 2005;17:701–6.
[34] Baldassarre H, Bordignon V. Laparoscopic ovum pick-up for in vitro embryo production from dairy bovine and buffalo calves. Anim Reprod 2018;15:191–6.
[35] Baldassarre H. Laparoscopic ovum pick-up followed by in vitro embryo production and transfer in assisted breeding programs for ruminants. Animals 2021;11:216.
[36] Taneja M, Bols PEJ, de Velde AV, Ju J-C, Schreiber D, Tripp MW, et al. Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotropin stimulation. Biol Reprod 2000;62:206–13.
[37] Romaguera R, Moll X, Morató R, Roura M, Palomo MJ, Catalá MG, et al. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. Theriogenology 2011;76:1–11.
[38] Liu K, Zhang L, Qi Q, Li J, Yan F, Hou J. Growth hormone treatment improves the development of follicles and oocytes in prepubertal lambs. J Ovarian Res 2023;16:132.
[39] Morton KM. Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes. Reprod Domest Anim 2008;43(Suppl 2):137–43.
[40] Morton KM, Catt SL, Maxwell WMC, Evans G. In vitro and in vivo developmental capabilities and kinetics of in vitro development of in vitro matured oocytes from adult, unstimulated and hormone-stimulated prepubertal ewes. Theriogenology 2005;64:1320–32.
[41] Baldassarre H, Currin L, Michalovic L, Bellefleur A-M, Gutierrez K, Mondadori RG, et al. Interval of gonadotropin administration for in vitro embryo production from oocytes collected from Holstein calves between 2 and 6 months of age by repeated laparoscopy. Theriogenology 2018;116:64–70.
[42] Armstrong D. Recent advances in superovulation of cattle. Theriogenology 1993;39:7–24.
[43] McGrice H, Kelly JM, Kleemann DO, Kind KL, Hampton AJ, Hannemann P, et al. Plasma anti-Müllerian hormone concentration as a predictive endocrine marker for selection of donor lambs to improve success in juvenile in vitro embryo transfer programs. Reprod Fertil Dev 2020;32:383–91.
[44] Krause ART, Dias FCF, Caunce SL, Adams GP, Mapletoft RJ, Singh J. Predictors of the ovarian superstimulatory response and oocyte collection in prepubertal heifers. Domest Anim Endocrinol 2022;81:106729.
[45] Currin L, Baldassarre H, Bordignon V. In vitro production of embryos from prepubertal holstein cattle and mediterranean water buffalo: Problems, progress and potential. Animals 2021;11:2275.
[46] Landry DA, Bellefleur A-M, Labrecque R, Grand F-X, Vigneault C, Blondin P, et al. Effect of cow age on the in vitro developmental competence of oocytes obtained after FSH stimulation and coasting treatments. Theriogenology 2016;86:1240–6.
[47] Li R, Albertini DF. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. Nat Rev Mol Cell Biol 2013;14:141–52.
[48] Damiani P, Fissore RA, Cibelli JB, Long CR, Balise JJ, Robl JM, et al. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Mol Reprod Dev 1996;45:521–34.
[49] Martino NA, Picardi E, Ciani E, D'Erchia AM, Bogliolo L, Ariu F, et al. Cumulus cell transcriptome after cumulus-oocyte complex exposure to nanomolar cadmium in an in vitro animal model of prepubertal and adult age. Biology 2023;12:249.
[50] Piras AR, Menéndez-Blanco I, Soto-Heras S, Catalá MG, Izquierdo D, Bogliolo L, et al. Resveratrol supplementation during in vitro maturation improves embryo development of prepubertal goat oocytes selected by brilliant cresyl blue staining. J Reprod Dev 2019;65:113–20.

[51] Ledda S, Bogliolo L, Calvia P, Leoni G, Naitana S. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J Reprod Fertil 1997;109:73–8.
[52] Nagano M, Katagiri S, Takahashi Y. Relationship between bovine oocyte morphology and in vitro developmental potential. Zygote 2006;14:53–61.
[53] Palmerini MG, Nottola SA, Leoni GG, Succu S, Borshi X, Berlinguer F, et al. In vitro maturation is slowed in prepubertal lamb oocytes: ultrastructural evidences. Reprod Biol Endocrinol 2014;12:115.
[54] Velilla E, RodrÍguez-Gonzalez E, Vidal F, Izquierdo D, Paramio M-T. Mitochondrial organization in prepubertal goat oocytes during in vitro maturation and fertilization. Mol Reprod Dev 2006;73:617–26.
[55] Brevini TA, Cillo F, Antonini S, Gandolfi F. Cytoplasmic remodelling and the acquisition of developmental competence in pig oocytes. Anim Reprod Sci 2007; 98:23–38.
[56] Salamone DF, Damiani P, Fissore RA, Robl JM, Duby RT. Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biol Reprod 2001;64:1761–8.
[57] Bernal-Ulloa SM, Heinzmann J, Herrmann D, Hadeler KG, Aldag P, Winkler S, et al. Cyclic AMP affects oocyte maturation and embryo development in prepubertal and adult cattle. PLoS One 2016;11:e0150264.
[58] Ying C, Yangsheng W, Jiapeng L, Liqin W, Xiaolin L, Mingjun L, et al. Transcriptome profiles of pre-pubertal and adult in vitro matured ovine oocytes obtained from FSH-stimulated animals. Reprod Domest Anim 2021;56:1085–94.
[59] da Silveira JC, de Ávila ACF, Garrett HL, Bruemmer JE, Winger QA, Bouma GJ. Cell-secreted vesicles containing microRNAs as regulators of gamete maturation. J Endocrinol 2018;236:R15–27.
[60] Dorji Ohkubo Y, Miyoshi K, Yoshida M. Gene expression differences in oocytes derived from adult and prepubertal Japanese Black cattle during in vitro maturation. Reprod Domest Anim 2012;47:392–402.
[61] Leoni GG, Bebbere D, Succu S, Berlinguer F, Mossa F, Galioto M, et al. Relations between relative mRNA abundance and developmental competence of ovine oocytes. Mol Reprod Dev 2007;74:249–57.
[62] Bebbere D, Ariu F, Bogliolo L, Masala L, Murrone O, Fattorini M, et al. Expression of maternally derived KHDC3, NLRP5, OOEP and TLE6 is associated with oocyte developmental competence in the ovine species. BMC Dev Biol 2014;14:40.
[63] Gandolfi F, Milanesi E, Pocar P, Luciano AM, Brevini TAL, Acocella F, et al. Comparative analysis of calf and cow oocytes during in vitro maturation. Mol Reprod Dev 1998;49:168–75.
[64] Pasquariello R, Manzoni EFM, Fiandanese N, Viglino A, Pocar P, Brevini TAL, et al. Implications of miRNA expression pattern in bovine oocytes and follicular fluids for developmental competence. Theriogenology 2020;145:77–85.
[65] De Santis L, Gandolfi F, Pennarossa G, Maffei S, Gismano E, Intra G, et al. Expression and intracytoplasmic distribution of staufen and calreticulin in maturing human oocytes. J Assist Reprod Genet 2015;32:645–52.
[66] Brevini TAL, Vassena R, Francisci C, Gandolfi F. Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. Biol Reprod 2005;72: 1218–23.
[67] Ledda S, Bogliolo L, Leoni G, Naitana S. Cell coupling and maturation-promoting factor activity in in vitro-matured prepubertal and adult sheep oocytes. Biol Reprod 2001;65:247–52.
[68] Ledda S, Bogliolo L, Leoni G, Calvia P, Naitana S. Influence of vasoactive intestinal peptide (VIP), atrial natriuretic peptide (ANP) and insulin-like growth factor-I (IGF-I) on in vitro maturation of prepubertal and adult sheep oocytes. Zygote 1996;4:343–8.
[69] Homa ST, Racowsky C, McGaughey RW. Lipid analysis of immature pig oocytes. J Reprod Fertil 1986;77:425–34.
[70] Abazarikia A, Ariu F, Rasekhi M, Zhandi M, Ledda S. Distribution and size of lipid droplets in oocytes recovered from young lamb and adult ovine ovaries. Reprod Fertil Dev 2020;32:1022–6.
[71] Warzych E, Pawlak P, Pszczola M, Cieslak A, Lechniak D. Prepubertal heifers versus cows-The differences in the follicular environment. Theriogenology 2017; 87:36–47.
[72] Del Collado M, da Silveira JC, Oliveira MLF, Alves B, Simas RC, Godoy AT, et al. In vitro maturation impacts cumulus-oocyte complex metabolism and stress in cattle. Reproduction 2017;154:881–93.
[73] Brusentsev EY, Mokrousova VI, Igonina TN, Rozhkova IN, Amstislavsky SY. Role of lipid droplets in the development of oocytes and preimplantation embryos in mammals. Russ J Dev Biol 2019;50:230–7.
[74] Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006;65:126–36.
[75] Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. Mol Reprod Dev 1994;37:48–53.
[76] Aguila L, Treulen F, Therrien J, Felmer R, Valdivia M. Oocyte selection for in vitro embryo production in bovine species: noninvasive approaches for new challenges of oocyte competence. Animals 2020;10.
[77] Warriach HM, Chohan KR. Thickness of cumulus cell layer is a significant factor in meiotic competence of buffalo oocytes. J Vet Sci 2004;5:247–51.
[78] Lasiene K, Vitkus A, Valanciüte A, Lasys V. Morphological criteria of oocyte quality. Medicina 2009;45:509–15.
[79] Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, et al. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. Reproduction 2005;130: 485–95.
[80] Gérard N, Loiseau S, Duchamp G, Seguin F. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 2002;124:241–8.
[81] Lemseffer Y, Terret M-E, Campillo C, Labrune E. Methods for assessing oocyte quality: a review of literature. Biomedicines 2022;10:2184.
[82] Revelli A, Piane LD, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 2009;7:1–13.
[83] Junior ARP, van Tilburg MF, Lobo MD, Monteiro-Moreira AC, Moreira RA, Melo CH, et al. Proteomic analysis of follicular fluid from tropically-adapted goats. Anim Reprod Sci 2018;188:35–44.
[84] Maniwa J, Izumi S, Isobe N, Terada T. Studies on substantially increased proteins in follicular fluid of bovine ovarian follicular cysts using 2-D PAGE and MALDI-TOF MS. Reprod Biol Endocrinol 2005;3:1–9.
[85] Panner Selvam MK, Baskaran S, Agarwal A. Chapter six - proteomics of reproduction: prospects and perspectives. In: Makowski GS, editor. Advances in clinical chemistry. Elsevier; 2019. p. 217–43.
[86] Twigt J, Steegers-Theunissen RP, Bezstarosti K, Demmers JA. Proteomic analysis of the microenvironment of developing oocytes. Proteomics 2012;12:1463–71.
[87] Fu Q, Huang Y, Wang Z, Chen F, Huang D, Lu Y, et al. Proteome profile and quantitative proteomic analysis of buffalo (Bubalusbubalis) follicular fluid during follicle development. Int J Mol Sci 2016;17:618.
[88] Wu Y, Lin J, Han B, Wang L, Chen Y, Liu M, et al. Proteomic profiling of follicle fluids after superstimulation in one-month-old lambs. Reprod Domest Anim 2018; 53:186–94.
[89] Khatir H, Lonergan P, Carolan C, Mermillod P. Prepubertal bovine oocyte: a negative model for studying oocyte developmental competence. Mol Reprod Dev 1996;45:231–9.
[90] Contreras-Solís I, Catalá M, Soto-Heras S, Roura M, Paramio MT, Izquierdo D. Effect of follicle size on hormonal status of follicular fluid, oocyte ATP content, and in vitro embryo production in prepubertal sheep. Domest Anim Endocrinol 2021;75:106582.
[91] Izquierdo D, Roura M, Pérez-Trujillo M, Soto-Heras S, Paramio MT. Fatty acids and metabolomic composition of follicular fluid collected from environments associated with good and poor oocyte competence in goats, vol. 23; 2022.
[92] Warzych E, Pawlak P, Pszczola M, Cieslak A, Lechniak D. Prepubertal heifers versus cows—the differences in the follicular environment. Theriogenology 2017; 87:36–47.
[93] Alves GP, Cordeiro FB, Bruna de Lima C, Annes K, Cristina Dos Santos É, Ispada J, et al. Follicular environment as a predictive tool for embryo development and kinetics in cattle. Reprod Fertil Dev 2019;31:451–61.
[94] Bender K, Walsh S, Evans A, Fair T, Brennan L. Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. Reproduction 2010;139:1047–55.
[95] Matthaus C, Bird B, Miljkovic M, Chernenko T, Romeo M, Diem M. Chapter 10: infrared and Raman microscopy in cell biology. Methods Cell Biol 2008;89: 275–308.
[96] Huang X, Hong L, Wu Y, Chen M, Kong P, Ruan J, et al. Raman spectrum of follicular fluid: a potential biomarker for oocyte developmental competence in polycystic ovary syndrome. Front Cell Dev Biol 2021;9:777224.
[97] Di Pietro C. Exosome-mediated communication in the ovarian follicle. J Assist Reprod Genet 2016;33:303–11.
[98] Donadeu FX, Schauer S, Sontakke S. Involvement of miRNAs in ovarian follicular and luteal development. J Endocrinol 2012;215:323.
[99] Hung W-T, Hong X, Christenson LK, McGinnis LK. Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biol Reprod 2015;93(117): 1–9.
[100] Machtinger R, Rodosthenous RS, Adir M, Mansour A, Racowsky C, Baccarelli AA, et al. Extracellular microRNAs in follicular fluid and their potential association with oocyte fertilization and embryo quality: an exploratory study. J Assist Reprod Genet 2017;34:525–33.
[101] Reza AMMT, Choi Y-J, Han SG, Song H, Park C, Hong K, et al. Roles of microRNAs in mammalian reproduction: from the commitment of germ cells to peri-implantation embryos. Biol Rev 2019;94:415–38.
[102] Nouri N, Shareghi-Oskoue O, Aghebati-Maleki L, Danaii S, Ahmadian Heris J, Soltani-Zangbar MS, et al. Role of miRNAs interference on ovarian functions and premature ovarian failure. Cell Commun Signal 2022;20:198.
[103] Zhang X, Dong C, Yang J, Li Y, Feng J, Wang B, et al. The roles of the miRNAome and transcriptome in the ovine ovary reveal poor efficiency in juvenile superovulation. Animals 2021;11.
[104] da Silveira JC, Andrade GM, Del Collado M, Sampaio RV, Sangalli JR, Silva LA, et al. Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One 2017;12:e0179451.
[105] Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 2002;296: 2178–80.
[106] Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction 2001;122:829–38.
[107] Bettegowda A, Patel OV, Lee K-B, Park K-E, Salem M, Yao J, et al. Identification of novel bovine cumulus cell molecular markers predictive of oocyte competence: functional and diagnostic implications. Biol Reprod 2008;79:301–9.
[108] Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Clinical assisted reproduction: cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF–ET. J Assist Reprod Genet 2001;18:490–8.

[109] Ikeda S, Imai H, Yamada M. Apoptosis in cumulus cells during in vitro maturation of bovine cumulus-enclosed oocytes. Reproduction 2003;125:369–76.
[110] Janowski D, Salilew-Wondim D, Torner H, Tesfaye D, Ghanem N, Tomek W, et al. Incidence of apoptosis and transcript abundance in bovine follicular cells is associated with the quality of the enclosed oocyte. Theriogenology 2012;78:656–69. e1-5.
[111] Bencomo E, Pérez R, Arteaga M-F, Acosta E, Peña O, Lopez L, et al. Apoptosis of cultured granulosa-lutein cells is reduced by insulin-like growth factor I and may correlate with embryo fragmentation and pregnancy rate. Fertil Steril 2006;85:474–80.
[112] Anguita B, Paramio MT, Morató R, Romaguera R, Jiménez-Macedo AR, Mogas T, et al. Effect of the apoptosis rate observed in oocytes and cumulus cells on embryo development in prepubertal goats. Anim Reprod Sci 2009;116:95–106.
[113] Warzych E, Pers-Kamczyc E, Krzywak A, Dudzińska S, Lechniak D. Apoptotic index within cumulus cells is a questionable marker of meiotic competence of bovine oocytes matured in vitro. Reprod Biol 2013;13:82–7.
[114] Seli E, Robert C, Sirard M-A. OMICS in assisted reproduction: possibilities and pitfalls. Mol Hum Reprod 2010;16:513–30.
[115] Burnik Papler T, Vrtačnik Bokal E, Maver A, Lovrečić L. Specific gene expression differences in cumulus cells as potential biomarkers of pregnancy. Reprod Biomed Online 2015;30:426–33.
[116] Uyar A, Torrealday S, Seli E. Cumulus and granulosa cell markers of oocyte and embryo quality. Fertil Steril 2013;99:979–97.
[117] Wathlet S, Adriaenssens T, Segers I, Verheyen G, Janssens R, Coucke W, et al. New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression. Fertil Steril 2012;98:432–9. e4.
[118] Fragouli E, Lalioti MD, Wells D. The transcriptome of follicular cells: biological insights and clinical implications for the treatment of infertility. Hum Reprod Update 2013;20:1–11.
[119] Tian H, Ren P, Liu K, Qiu C, Fan L, Li J, et al. Transcriptomic comparison of ovarian granulosa cells between adult sheep and prepubertal lambs. BMC Genom 2022;23:151.
[120] Lin J, Wu Y, Han B, Chen Y, Wang L, Li X, et al. iTRAQ-based proteomic profiling of granulosa cells from lamb and Ewe after superstimulation. Theriogenology 2017;101:99–108.
[121] Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004;82–83:431–46.
[122] Tian H, Ren P, Liu K, Qiu C, Fan L, Li J, et al. Transcriptomic comparison of ovarian granulosa cells between adult sheep and prepubertal lambs. BMC Genom 2022;23:151.
[123] Tan TCY, Dunning KR. Non-invasive assessment of oocyte developmental competence. Reprod Fertil Dev 2022;35:39–50.
[124] O'Brien J, Dwarte D, Ryan J, Maxwell W, Evans G. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. Reprod Fertil Dev 1996;8:1029–37.
[125] Hemmings KE, Leese HJ, Picton HM. Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. Biol Reprod 2012;86(165):1–12.
[126] Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol Reprod 2010;83:909–18.
[127] Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006;73:1195–201.
[128] Tan TC, Brown HM, Thompson JG, Mustafa S, Dunning KR. Optical imaging detects metabolic signatures associated with oocyte quality. Biol Reprod 2022;107:1014–25.
[129] Rusciano G, De Canditiis C, Zito G, Rubessa M, Roca MS, Carotenuto R, et al. Raman-microscopy investigation of vitrification-induced structural damages in mature bovine oocytes. PLoS One 2017;12:e0177677.
[130] Monteiro CAS, Chow DJ, Leal GR, Tan TC, Ferreira AMR, Thompson JG, et al. Optical imaging of cleavage stage bovine embryos using hyperspectral and confocal approaches reveals metabolic differences between on-time and fast-developing embryos. Theriogenology 2021;159:60–8.
[131] Bogliolo L, Ledda S, Innocenzi P, Ariu F, Bebbere D, Rosati I, et al. Raman microspectroscopy as a non-invasive tool to assess the vitrification-induced changes of ovine oocyte zona pellucida. Cryobiology 2012;64:267–72.
[132] Bogliolo L, Murrone O, Piccinini M, Ariu F, Ledda S, Tilocca S, et al. Evaluation of the impact of vitrification on the actin cytoskeleton of in vitro matured ovine oocytes by means of Raman microspectroscopy. J Assist Reprod Genet 2015;32:185–93.
[133] Bogliolo L, Leoni GG, Ledda S. Raman spectroscopy-based approach to study the female gamete. Theriogenology 2020;150:268–75.
[134] McLennan H, Saini A, Dunning K, Thompson J. Oocyte and embryo evaluation by AI and multi-spectral auto-fluorescence imaging: livestock embryology needs to catch-up to clinical practice. Theriogenology 2020;150:255–62.
[135] Sanchez T, Venturas M, Aghvami SA, Yang X, Fraden S, Sakkas D, et al. Combined noninvasive metabolic and spindle imaging as potential tools for embryo and oocyte assessment. Hum Reprod 2019;34:2349–61.
[136] Perevedentseva E, Krivokharchenko A, Karmenyan AV, Chang H-H, Cheng C-L. Raman spectroscopy on live mouse early embryo while it continues to develop into blastocyst in vitro. Sci Rep 2019;9:6636.
[137] Jimenez LE, Juarez AC, Roldan-Olarte M, Alvarez RMS. Biochemical changes in the cytoplasm of bovine oocytes during the in vitro maturation process: a Raman microscopy study. Vet Res Commun 2022;46:549–62.
[138] Heraud P, Marzec KM, Zhang QH, Yuen WS, Carroll J, Wood BR. Label-free in vivo Raman microspectroscopic imaging of the macromolecular architecture of oocytes. Sci Rep 2017;7:8945.
[139] Jasensky J, Boughton AP, Khmaladze A, Ding J, Zhang C, Swain JE, et al. Live-cell quantification and comparison of mammalian oocyte cytosolic lipid content between species, during development, and in relation to body composition using nonlinear vibrational microscopy. Analyst 2016;141:4694–706.
[140] Bradley J, Pope I, Masia F, Sanusi R, Langbein W, Swann K, et al. Quantitative imaging of lipids in live mouse oocytes and early embryos using CARS microscopy. Development 2016;143:2238–47.
[141] Bradley J, Pope I, Wang Y, Langbein W, Borri P, Swann K. Dynamic label-free imaging of lipid droplets and their link to fatty acid and pyruvate oxidation in mouse eggs. J Cell Sci 2019;132.
[142] Venturas M, Yang X, Sakkas D, Needleman D. Noninvasive metabolic profiling of cumulus cells, oocytes, and embryos via fluorescence lifetime imaging microscopy: a mini-review. Hum Reprod 2023;38:799–810.
[143] Ericsson S. Assessment of porcine oocytes using brilliant cresyl blue. Theriogenology 1993;39:214.
[144] Opiela J, Katska-Ksiazkiewicz L. The utility of Brilliant Cresyl Blue (BCB) staining of mammalian oocytes used for in vitro embryo production (IVP). Reprod Biol 2013;13:177–83.
[145] Rodrıguez-González E, Lopez-Bejar M, Velilla E, Paramio M. Selection of prepubertal goat oocytes using the brilliant cresyl blue test. Theriogenology 2002;57:1397–409.
[146] Rodríguez-González E, López-Bejar M, Mertens MJ, Paramio MT. Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. Mol Reprod Dev 2003;65:446–53.
[147] Catalá MG, Izquierdo D, Uzbekova S, Morató R, Roura M, Romaguera R, et al. Brilliant Cresyl Blue stain selects largest oocytes with highest mitochondrial activity, maturation-promoting factor activity and embryo developmental competence in prepubertal sheep. Reproduction 2011;142:517.
[148] Gilchrist RB, Luciano AM, Richani D, Zeng HT, Wang X, Vos MD, et al. Oocyte maturation and quality: role of cyclic nucleotides. Reproduction 2016;152:R143–57.
[149] Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. Theriogenology 2007;67:6–15.
[150] Richani D, Gilchrist RB. Approaches to oocyte meiotic arrest in vitro and impact on oocyte developmental competence. Biol Reprod 2022;106:243–52.
[151] Albuz F, Sasseville M, Lane M, Armstrong D, Thompson J, Gilchrist R. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. Hum Reprod 2010;25:2999–3011.
[152] Ali A, Sirard M-A. Protein kinases influence bovine oocyte competence during short-term treatment with recombinant human follicle stimulating hormone. Reproduction 2005;130:303–10.
[153] Bernal SM, Heinzmann J, Herrmann D, Timmermann B, Baulain U, Großfeld R, et al. Effects of different oocyte retrieval and in vitro maturation systems on bovine embryo development and quality. Zygote 2015;23:367–77.
[154] Buell M, Chitwood JL, Ross PJ. cAMP modulation during sheep in vitro oocyte maturation delays progression of meiosis without affecting oocyte parthenogenetic developmental competence. Anim Reprod Sci 2015;154:16–24.
[155] Franciosi F, Coticchio G, Lodde V, Tessaro I, Modina SC, Fadini R, et al. Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. Biol Reprod 2014;91:61.
[156] Guimarães A, Pereira S, Leme L, Dode M. Evaluation of the simulated physiological oocyte maturation system for improving bovine in vitro embryo production. Theriogenology 2015;83:52–7.
[157] Li H, Sutton-McDowall M, Wang X, Sugimura S, Thompson J, Gilchrist R. Extending prematuration with cAMP modulators enhances the cumulus contribution to oocyte antioxidant defence and oocyte quality via gap junctions. Hum Reprod 2016;31:810–21.
[158] Lodde V, Franciosi F, Tessaro I, Modina SC, Luciano AM. Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. J Assist Reprod Genet 2013;30:1219–26.
[159] Luciano AM, Franciosi F, Modina SC, Lodde V. Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s). Biol Reprod 2011;85:1252–9.
[160] Luciano AM, Pocar P, Milanesi E, Modina S, Rieger D, Lauria A, et al. Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. Mol Reprod Dev 1999;54:86–91.
[161] Rose RD, Gilchrist RB, Kelly JM, Thompson JG, Sutton-McDowall ML. Regulation of sheep oocyte maturation using cAMP modulators. Theriogenology 2013;79:142–8.
[162] Xi G, An L, Jia Z, Tan K, Zhang J, Wang Z, et al. Natriuretic peptide receptor 2 (NPR2) localized in bovine oocyte underlies a unique mechanism for C-type natriuretic peptide (CNP)-induced meiotic arrest. Theriogenology 2018;106:198–209.
[163] Soto-Heras S, Menéndez-Blanco I, Catalá M-G, Izquierdo D, Thompson JG, Paramio M-T. Biphasic in vitro maturation with C-type natriuretic peptide

enhances the developmental competence of juvenile-goat oocytes. PLoS One 2019;14:e0221663.
[164] Tscharke MT, Kelly JM, Kind KL. Effect of a pre-treatment with C-type natriuretic peptide and estradiol on developmental competence of lamb oocytes. Anim Sci Proceed 2023;14:451–2.
[165] Wang L, Jiang X, Wu Y, Lin J, Zhang L, Yang N, et al. Effect of milrinone on the developmental competence of growing lamb oocytes identified with brilliant cresyl blue. Theriogenology 2016;86:2020–7.
[166] Romero S, Sánchez F, Lolicato F, Van Ranst H, Smitz J. Immature oocytes from unprimed juvenile mice become a valuable source for embryo production when using C-type natriuretic peptide as essential component of culture medium. Biol Reprod 2016;95(64):1–10.
[167] Soto-Heras S, Paramio M-T. Impact of oxidative stress on oocyte competence for in vitro embryo production programs. Res Vet Sci 2020;132:342–50.
[168] Jiao G-Z, Cao X-Y, Cui W, Lian H-Y, Miao Y-L, Wu X-F, et al. Developmental potential of prepubertal mouse oocytes is compromised due mainly to their impaired synthesis of glutathione. PLoS One 2013;8:e58018.
[169] Yuan Y, Wheeler MB, Krisher RL. Disrupted redox homeostasis and aberrant redox gene expression in porcine oocytes contribute to decreased developmental competence. Biol Reprod 2012;87.
[170] Khazaei M, Aghaz F. Reactive oxygen species generation and use of antioxidants during in vitro maturation of oocytes. Int J Fertil Steril 2017;11:63–70.
[171] Deleuze S, Goudet G. Cysteamine supplementation of in vitro maturation media: a review. Reprod Domest Anim 2010;45:e476–82.
[172] Kelly JM, Kleemann DO, Maxwell WM, Walker SK. Effects of insulin-like growth factor-I, epidermal growth factor and cysteamine on the in vitro maturation and development of oocytes collected from 6- to 8-week-old Merino lambs. Reprod Fertil Dev 2008;20:570–8.
[173] Rodríguez-González E, López-Bejar M, Izquierdo D, Paramio M-T. Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation. Reprod Nutr Dev 2003;43:179–87.
[174] Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC, Sainz RM. Melatonin and reproduction revisited. Biol Reprod 2009;81:445–56.
[175] Soto-Heras S, Catalá MG, Roura M, Menéndez-Blanco I, Piras AR, Izquierdo D, et al. Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats. Reprod Domest Anim 2019;54:381–90.
[176] Gutiérrez-Añez JC, Lucas-Hahn A, Hadeler K-G, Aldag P, Niemann H. Melatonin enhances in vitro developmental competence of cumulus-oocyte complexes collected by ovum pick-up in prepubertal and adult dairy cattle. Theriogenology 2021;161:285–93.
[177] Tian H, Qi Q, Yan F, Wang C, Hou F, Ren W, et al. Enhancing the developmental competence of prepubertal lamb oocytes by supplementing the in vitro maturation medium with sericin and the fibroblast growth factor 2-leukemia inhibitory factor-insulin-like growth factor 1 combination. Theriogenology 2021;159:13–9.
[178] Pasquariello R, Verdile N, Brevini TAL, Gandolfi F, Boiti C, Zerani M, et al. The role of resveratrol in mammalian reproduction. Molecules 2020;25.
[179] De Oliveira MR, Nabavi SF, Manayi A, Daglia M, Hajheydari Z, Nabavi SM. Resveratrol and the mitochondria: from triggering the intrinsic apoptotic pathway to inducing mitochondrial biogenesis, a mechanistic view. Biochim Biophys Acta Gen Subj 2016;1860:727–45.
[180] Sprícigo JF, Morató R, Arcarons N, Yeste M, Dode MA, Lopez-Bejar M, et al. Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. Theriogenology 2017;89:47–57.
[181] Petridis A, Therios I, Samouris G, Tananaki C. Salinity-induced changes in phenolic compounds in leaves and roots of four olive cultivars (Olea europaea L.) and their relationship to antioxidant activity. Environ Exp Bot 2012;79:37–43.
[182] Martino NA, Ariu F, Bebbere D, Uranio MF, Chirico A, Marzano G, et al. Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: a large animal model study. Reprod Toxicol 2016;65:204–11.
[183] Dell'Aquila ME, Bogliolo L, Russo R, Martino NA, Filioli Uranio M, Ariu F, et al. Prooxidant effects of verbascoside, a bioactive compound from olive oil mill wastewater, on in vitro developmental potential of ovine prepubertal oocytes and bioenergetic/oxidative stress parameters of fresh and vitrified oocytes. BioMed Res Int 2014;2014:878062.
[184] Tao W, Li M, Xie R. Preparation and structure of porous silk sericin materials. Macromol Mater Eng 2005;290:188–94.
[185] Takahashi M, Tsujimoto K, Kato Y, Yamada H, Takagi H, Nakamori S. A sericin-derived peptide protects sf9 insect cells from death caused by acute serum deprivation. Biotechnol Lett 2005;27:893–7.
[186] Ariu F, Bogliolo L, Pinna A, Malfatti L, Innocenzi P, Falchi L, et al. Cerium oxide nanoparticles (CeO2 NPs) improve the developmental competence of *in vitro*-matured prepubertal ovine oocytes. Reprod Fertil Dev 2017;29:1046–56.
[187] Izquierdo D, Roura M, Pérez-Trujillo M, Soto-Heras S, Paramio M-T. Fatty acids and metabolomic composition of follicular fluid collected from environments associated with good and poor oocyte competence in goats. Int J Mol Sci 2022;23:4141.
[188] Driancourt MA, Reynaud K, Smitz J. Differences in follicular function of 3-month-old calves and mature cows. Reproduction 2001;121:463–74.
[189] Tian H, Liu K, Zhang Y, Qi Q, Wang C, Guan H, et al. Adult follicular fluid supplementation during in vitro maturation improves the developmental competence of prepubertal lamb oocytes. Theriogenology 2019;130:157–62.
[190] Ghaffarilaleh V, Fouladi-Nashta A, Paramio M-T. Effect of α-linolenic acid on oocyte maturation and embryo development of prepubertal sheep oocytes. Theriogenology 2014;82:686–96.
[191] Brevini TA, Pennarossa G, Gandolfi F. A 3D approach to reproduction. Theriogenology 2020;150:2–7.
[192] Park JE, Kim MS, Lee E, Lee ST. In vitro maturation using an agarose matrix with incorporated extracellular matrix proteins improves porcine oocyte developmental competence by enhancing cytoplasmic maturation. J Tissue Eng Regen Med 2021;15:807–17.
[193] Shen P, Xu J, Wang P, Zhao X, Huang B, Wu F, et al. A new three-dimensional glass scaffold increases the in vitro maturation efficiency of buffalo (Bubalus bubalis) oocyte via remodelling the extracellular matrix and cell connection of cumulus cells. Reprod Domest Anim 2020;55:170–80.
[194] Mastrorocco A, Cacopardo L, Martino NA, Fanelli D, Camillo F, Ciani E, et al. One-step automated bioprinting-based method for cumulus-oocyte complex microencapsulation for 3D in vitro maturation. PLoS One 2020;15:e0238812.
[195] Dorati R, Genta I, Ferrari M, Vigone G, Merico V, Garagna S, et al. Formulation and stability evaluation of 3D alginate beads potentially useful for cumulus–oocyte complexes culture. J Microencapsul 2016;33:137–45.
[196] Mastrorocco A, Cacopardo L, Lamanna D, Temerario L, Brunetti G, Carluccio A, et al. Bioengineering approaches to improve in vitro performance of prepubertal lamb oocytes. Cells 2021;10:1458.
[197] Mastrorocco A, Cacopardo L, Temerario L, Martino NA, Tridente F, Rizzo A, et al. Investigating and modelling an engineered millifluidic in vitro oocyte maturation system reproducing the physiological ovary environment in the sheep model. Cells 2022;11:3611.
[198] Aussillous P, Quéré D. Liquid marbles. Nature 2001;411:924–7.
[199] Bebbere D, Nieddu SM, Ariu F, Piras D, Ledda S. 3D liquid marble microbioreactors support in vitro maturation of prepubertal ovine oocytes and affect expression of oocyte-specific factors. Biology 2021;10:1101.
[200] Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 1977;74:68–85.
[201] Willison KR, Hynes G, Davies P, Goldsborough A, Lewis VA. Expression of three t-complex genes, Tcp-1, D17Leh117c3, and D17Leh66, in purified murine spermatogenic cell populations. Genet Res 1990;56:193–201.
[202] Wolgemuth DJ, Gizang-Ginsberg E, Engelmyer E, Gavin BJ, Ponzetto C. Separation of mouse testis cells on a Celsep (TM) apparatus and their usefulness as a source of high molecular weight DNA or RNA. Gamete Res 1985;12:1–10.
[203] Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews Jr DH, Boisclair YR, et al. Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. Reproduction 2007;134:171–81.
[204] Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews Jr DH, Mir PS, et al. Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. Domest Anim Endocrinol 2007;33:460–9.
[205] Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews Jr DH, Mir PS, et al. Effect of nutrition during calfhood and peripubertal period on serum metabolic hormones, gonadotropins and testosterone concentrations, and on sexual development in bulls. Domest Anim Endocrinol 2007;33:1–18.
[206] Byrne TJ, Santos BFS, Amer PR, Martin-Collado D, Pryce JE, Axford M. New breeding objectives and selection indices for the Australian dairy industry. J Dairy Sci 2016;99:8146–67.
[207] Dance A, Thundathil J, Wilde R, Blondin P, Kastelic J. Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. J Dairy Sci 2015;98:987–98.
[208] Harstine BR, Maquivar M, Helser LA, Utt MD, Premanandan C, DeJarnette JM, et al. Effects of dietary energy on sexual maturation and sperm production in Holstein bulls. J Anim Sci 2015;93:2759–66.
[209] Cremades N, Bernabeu R, Barros A, Sousa M. In-vitro maturation of round spermatids using co-culture on Vero cells. Hum Reprod 1999;14:1287–93.
[210] Tanaka A, Nagayoshi M, Awata S, Mawatari Y, Tanaka I, Kusunoki H. Completion of meiosis in human primary spermatocytes through in vitro coculture with Vero cells. Fertil Steril 2003;79(Suppl 1):795–801.
[211] Tesarik J, Bahceci M, Özcan C, Greco E, Mendoza C. Restoration of fertility by in-vitro spermatogenesis. Lancet 1999;353:555–6.
[212] Tesarik J, Greco E, Rienzi L, Ubaldi F, Guido M, Cohen-Bacrie P, et al. Differentiation of spermatogenic cells during in-vitro culture of testicular biopsy samples from patients with obstructive azoospermia: effect of recombinant follicle stimulating hormone. Hum Reprod 1998;13:2772–81.
[213] Tesarik J, Guido M, Mendoza C, Greco E. Human spermatogenesis in vitro: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis. J Clin Endocrinol Metab 1998;83:4467–73.
[214] Movahedin M, Ajeen A, Ghorbanzadeh N, Tiraihi T, Valojerdi MR, Kazemnejad A. In vitro maturation of fresh and frozen-thawed mouse round spermatids. Andrologia 2004;36:269–76.
[215] Fujihara M, Kim SM, Minami N, Yamada M, Imai H. Characterization and in vitro culture of male germ cells from developing bovine testis. J Reprod Dev 2011;57:355–64.
[216] Izadyar F, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, De Rooij DG. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. Biol Reprod 2003;68:272–81.

[217] Reding SC, Stepnoski AL, Cloninger EW, Oatley JM. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. Reproduction 2010;139:893–903.
[218] Oatley MJ, Kaucher AV, Yang QE, Waqas MS, Oatley JM. Conditions for long-term culture of cattle undifferentiated spermatogonia. Biol Reprod 2016;95c:14.
[219] Zhao X, Wan W, Li B, Zhang X, Zhang M, Wu Z, et al. Isolation and in vitro expansion of porcine spermatogonial stem cells. Reprod Domest Anim 2022;57:210–20.
[220] Costa GMJ, Avelar GF, Lacerda S, Figueiredo AFA, Tavares AO, Rezende-Neto JV, et al. Horse spermatogonial stem cell cryopreservation: feasible protocols and potential biotechnological applications. Cell Tissue Res 2017;370:489–500.
[221] Xie B, Qin Z, Huang B, Xie T, Yao H, Wei Y, et al. In vitro culture and differentiation of buffalo (Bubalus bubalis) spermatogonia. Reprod Domest Anim 2010;45:275–82.
[222] Sakib S, Uchida A, Valenzuela-Leon P, Yu Y, Valli-Pulaski H, Orwig K, et al. Formation of organotypic testicular organoids in microwell culture. Biol Reprod 2019;100:1648–60.
[223] Wang D, Hildorf S, Ntemou E, Mamsen LS, Dong L, Pors SE, et al. Organotypic culture of testicular tissue from infant boys with cryptorchidism. Int J Mol Sci 2022;23.
[224] von Kopylow K, Schulze W, Salzbrunn A, Schaks M, Schäfer E, Roth B, et al. Dynamics, ultrastructure and gene expression of human in vitro organized testis cells from testicular sperm extraction biopsies. Mol Hum Reprod 2018;24:123–34.
[225] Sun M, Yuan Q, Niu M, Wang H, Wen L, Yao C, et al. Efficient generation of functional haploid spermatids from human germline stem cells by three-dimensional-induced system. Cell Death Differ 2018;25:749–66.
[226] Richer G, Baert Y, Goossens E. In-vitro spermatogenesis through testis modelling: toward the generation of testicular organoids. Andrology 2020;8:879–91.
[227] Perrard MH, Sereni N, Schluth-Bolard C, Blondet A, Sg DE, Plotton I, et al. Complete human and rat ex vivo spermatogenesis from fresh or frozen testicular tissue. Biol Reprod 2016;95:89.
[228] Horvath-Pereira BO, Almeida G, da Silva Junior LN, do Nascimento PG, Horvath Pereira BO, Fireman J, et al. Biomaterials for testicular bioengineering: how far have we come and where do we have to go? Front Endocrinol 2023;14:1085872.
[229] Lee DR, Kaproth MT, Parks JE. In vitro production of haploid germ cells from fresh or frozen-thawed testicular cells of neonatal bulls. Biol Reprod 2001;65:873–8.
[230] Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med 2007;13:1219–27.
[231] Bi Y, Stuelten CH, Kilts T, Wadhwa S, Iozzo RV, Robey PG, et al. Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. J Biol Chem 2005;280:30481–9.
[232] Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: a dynamic view. Dev Biol 2010;341:126–40.
[233] Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000;287:1427–30.
[234] Ashouri Movassagh S, Ashouri Movassagh S, Banitalebi Dehkordi M, Pourmand G, Gholami K, Talebi A, et al. Isolation, identification and differentiation of human spermatogonial cells on three-dimensional decellularized sheep testis. Acta Histochem 2020;122:151623.
[235] Hayashi S, Yang J, Christenson L, Yanagimachi R, Hecht NB. Mouse preimplantation embryos developed from oocytes injected with round spermatids or spermatozoa have similar but distinct patterns of early messenger RNA expression. Biol Reprod 2003;69:1170–6.
[236] Ishikura Y, Ohta H, Sato T, Murase Y, Yabuta Y, Kojima Y, et al. In vitro reconstitution of the whole male germ-cell development from mouse pluripotent stem cells. Cell Stem Cell 2021;28:2167–21679. e9.
[237] Zhou Q, Wang M, Yuan Y, Wang X, Fu R, Wan H, et al. Complete meiosis from embryonic stem cell-derived germ cells in vitro. Cell Stem Cell 2016;18:330–40.
[238] Ciccarelli M, Giassetti MI, Miao D, Oatley MJ, Robbins C, Lopez-Biladeau B, et al. Donor-derived spermatogenesis following stem cell transplantation in sterile *NANOS2* knockout males. Proc Natl Acad Sci USA 2020;117:24195–204.
[239] Herrid M, Olejnik J, Jackson M, Suchowerska N, Stockwell S, Davey R, et al. Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod 2009;81:898–905.
[240] Bogliotti YS, Wu J, Vilarino M, Okamura D, Soto DA, Zhong C, et al. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. Proc Natl Acad Sci USA 2018;115:2090–5.
[241] Gandolfi F, Pennarossa G, Maffei S, Brevini T. Why is it so difficult to derive pluripotent stem cells in domestic ungulates? Reprod Domest Anim 2012;47(Suppl 5):11–7.
[242] Meistrich ML. Separation of spermatogenic cells and nuclei from rodent testes. Methods Cell Biol 1977;15:15–54.
[243] Blanchard Y, Lavault MT, Quernee D, Le Lannou D, Lobel B, Lescoat D. Preparation of spermatogenic cell populations at specific stages of differentiation in the human. Mol Reprod Dev 1991;30:275–82.
[244] Pelengaris SA, Moore HD. Separation of round spermatids from the rat using an immunoselection panning technique. Mol Reprod Dev 1995;41:348–54.
[245] Ock SA, Kwack DO, Lee SL, Cho SR, Jeon BG, Kumar BM, et al. In vitro development of bovine oocytes reconstructed with round spermatids. Theriogenology 2006;65:1242–53.
[246] Kim CR, Noda T, Okada Y, Ikawa M, Baek SH. Protocol for isolation of spermatids from mouse testes. STAR Protoc 2021;2:100254.
[247] Lassalle B, Ziyyat A, Testart J, Finaz C, Lefevre A. Flow cytometric method to isolate round spermatids from mouse testis. Hum Reprod 1999;14:388–94.
[248] Hayama T, Yamaguchi T, Kato-Itoh M, Ishii Y, Mizuno N, Umino A, et al. Practical selection methods for rat and mouse round spermatids without DNA staining by flow cytometric cell sorting. Mol Reprod Dev 2016;83:488–96.
[249] Simard O, Leduc F, Acteau G, Arguin M, Gregoire MC, Brazeau MA, et al. Step-specific sorting of mouse spermatids by flow cytometry. J Vis Exp 2015:e53379.
[250] Aslam I, Robins A, Dowell K, Fishel S. Isolation, purification and assessment of viability of spermatogenic cells from testicular biopsies of azoospermic men. Hum Reprod 1998;13:639–45.
[251] Lacy D, Rotblat J. Study of normal and irradiated boundary tissue of the seminiferous tubules of the rat. Exp Cell Res 1960;21:49–70.
[252] Gardner PJ. Fine structure of the seminiferous tubule of the Swiss mouse. The spermatid. Anat Rec 1966;155:235–49.
[253] Tanaka A, Nagayoshi M, Takemoto Y, Tanaka I, Kusunoki H, Watanabe S, et al. Fourteen babies born after round spermatid injection into human oocytes. Proc Natl Acad Sci USA 2015;112:14629–34.
[254] Martins CF, Silva AE, Dode MN, Rumpf R, Cumpa HC, Silva CG, et al. Morphological characterization and conservation of bovine spermatogenic cells by refrigeration at 4 degrees C and freezing using different cryoprotective molecules. Cryobiology 2015;71:47–53.
[255] Goto K, Kinoshita A, Nakanishi Y, Ogawa K. Blastocyst formation following intracytoplasmic injection of in-vitro derived spermatids into bovine oocytes. Hum Reprod 1996;11:824–9.
[256] Bedford JM, Nicander L. Ultrastructural changes in the acrosome and sperm membranes during maturation of spermatozoa in the testis and epididymis of the rabbit and monkey. J Anat 1971;108:527–43.
[257] Aalseth EP, Saacke RG. Morphological change of the acrosome on motile bovine spermatozoa due to storage at 4 degrees C. J Reprod Fertil 1985;74:473–8.
[258] Reyes JG, Diaz A, Osses N, Opazo C, Benos DJ. On stage single cell identification of rat spermatogenic cells. Biol Cell 1997;89:53–66.
[259] Sutovsky P, Ramalho-Santos Jo, Moreno RD, Oko R, Hewitson L, Schatten G. On-stage selection of single round spermatids using a vital, mitochondrion-specific fluorescent probe MitoTracker™ and high resolution differential interference contrast microscopy. Hum Reprod 1999;14:2301–12.
[260] Okuda H, Tsujimura A, Yamamoto K, Fukuhara S, Nakayama J, Kiuchi H, et al. Morphologic and mitochondrial characterization of human spermatogenic cells dispersed in wet preparation for testicular sperm extraction: establishment of a microscopic diagram of developing human spermatogenic cells. Fertil Steril 2011;95:2665–8.
[261] Hikichi T, Kishigami S, Thuan NV, Ohta H, Mizutani E, Wakayama S, et al. Round spermatids stained with mitotracker can be used to produce offspring more simply. Zygote 2005;13:55–61.
[262] Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. Spermatid-specific expression of protamine 1 in transgenic mice. Proc Natl Acad Sci USA 1987;84:5316–9.
[263] Reddi PP, Flickinger CJ, Herr JC. Round spermatid-specific transcription of the mouse SP-10 gene is mediated by a 294-base pair proximal promoter. Biol Reprod 1999;61:1256–66.
[264] Iwanami Y, Kobayashi T, Kato M, Hirabayashi M, Hochi S. Characteristics of rat round spermatids differentiated from spermatogonial cells during co-culture with Sertoli cells, assessed by flow cytometry, microinsemination and RT-PCR. Theriogenology 2006;65:288–98.
[265] Bradshaw AW, Nikmehr B, Halicigil C, Stogner-Underwood K, Sadri-Ardekani H. Optimum identification of round spermatid in men with non-obstructive azoospermia: a commentary. Andrology 2021;9:1817–8.
[266] D'Occhio MJ, Hengstberger KJ, Johnston SD. Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim Reprod Sci 2007;101:1–17.
[267] Ren M, Xu Y, Erdjument-Bromage H, Donelian A, Phoon CKL, Terada N, et al. Extramitochondrial cardiolipin suggests a novel function of mitochondria in spermatogenesis. J Cell Biol 2019;218:1491–502.
[268] Li X, Duan C, Li R, Wang D. Insights into the mechanism of bovine spermiogenesis based on comparative transcriptomic studies. Animals 2021;11.
[269] Kleene KC. Position-dependent interactions of Y-box protein 2 (YBX2) with mRNA enable mRNA storage in round spermatids by repressing mRNA translation and blocking translation-dependent mRNA decay. Mol Reprod Dev 2016;83:190–207.
[270] Orsi GA, Couble P, Loppin B. Epigenetic and replacement roles of histone variant H3.3 in reproduction and development. Int J Dev Biol 2009;53:231–43.
[271] Bramlage B, Kosciessa U, Doenecke D. Differential expression of the murine histone genes H3.3A and H3.3B. Differentiation 1997;62:13–20.
[272] Couldrey C, Carlton MB, Nolan PM, Colledge WH, Evans MJ. A retroviral gene trap insertion into the histone 3.3A gene causes partial neonatal lethality, stunted growth, neuromuscular deficits and male sub-fertility in transgenic mice. Hum Mol Genet 1999;8:2489–95.
[273] van der Heijden GW, Derijck AA, Posfai E, Giele M, Pelczar P, Ramos L, et al. Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation. Nat Genet 2007;39:251–8.
[274] Thakar A, Gupta P, Ishibashi T, Finn R, Silva-Moreno B, Uchiyama S, et al. H2A.Z and H3.3 histone variants affect nucleosome structure: biochemical and biophysical studies. Biochemistry 2009;48:10852–7.
[275] Chen YS, Qiu XB. Transcription-coupled replacement of histones: degradation or recycling? J Genet Genomics 2012;39:575–80.
[276] Tang B, Yang Y, Wang G, Yao Z, Zhang L, Wu HC. A simple fluorescent probe based on a pyrene derivative for rapid detection of protamine and monitoring of trypsin activity. Org Biomol Chem 2015;13:8708–12.
[277] Meistrich ML, Bucci LR, Trostle-Weige PK, Brock WA. Histone variants in rat spermatogonia and primary spermatocytes. Dev Biol 1985;112:230–40.

[278] Montellier E, Boussouar F, Rousseaux S, Zhang K, Buchou T, Fenaille F, et al. Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. Genes Dev 2013;27:1680–92.
[279] Shinagawa T, Huynh LM, Takagi T, Tsukamoto D, Tomaru C, Kwak HG, et al. Disruption of Th2a and Th2b genes causes defects in spermatogenesis. Development 2015;142:1287–92.
[280] Drabent B, Bode C, Doenecke D. Structure and expression of the mouse testicular H1 histone gene (H1t). Biochim Biophys Acta 1993;1216:311–3.
[281] Drabent B, Kardalinou E, Bode C, Doenecke D. Association of histone H4 genes with the mammalian testis-specific H1t histone gene. DNA Cell Biol 1995;14:591–7.
[282] Grimes SR, Wolfe SA, Koppel DA. Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone H1t gene. Arch Biochem Biophys 1992;296:402–9.
[283] De Lucia F, Faraone-Mennella MR, D'Erme M, Quesada P, Caiafa P, Farina B. Histone-induced condensation of rat testis chromatin: testis-specific H1t versus somatic H1 variants. Biochem Biophys Res Commun 1994;198:32–9.
[284] Khadake JR, Rao MR. Condensation of DNA and chromatin by an SPKK-containing octapeptide repeat motif present in the C-terminus of histone H1. Biochemistry 1997;36:1041–51.
[285] Drabent B, Saftig P, Bode C, Doenecke D. Spermatogenesis proceeds normally in mice without linker histone H1t. Histochem Cell Biol 2000;113:433–42.
[286] Fantz DA, Hatfield WR, Horvath G, Kistler MK, Kistler WS. Mice with a targeted disruption of the H1t gene are fertile and undergo normal changes in structural chromosomal proteins during spermiogenesis. Biol Reprod 2001;64:425–31.
[287] Catena R, Ronfani L, Sassone-Corsi P, Davidson I. Changes in intranuclear chromatin architecture induce bipolar nuclear localization of histone variant H1T2 in male haploid spermatids. Dev Biol 2006;296:231–8.
[288] Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M, et al. Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. Proc Natl Acad Sci USA 2005;102:2808–13.
[289] Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, et al. HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. Mol Cell Biol 2005;25:7107–19.
[290] Yan W, Ma L, Burns KH, Matzuk MM. HILS1 is a spermatid-specific linker histone H1-like protein implicated in chromatin remodeling during mammalian spermiogenesis. Proc Natl Acad Sci USA 2003;100:10546–51.
[291] Zhong J, Peters AH, Kafer K, Braun RE. A highly conserved sequence essential for translational repression of the protamine 1 messenger rna in murine spermatids. Biol Reprod 2001;64:1784–9.
[292] Corzett M, Mazrimas J, Balhorn R. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 2002;61:519–27.
[293] Maier WM, Nussbaum G, Domenjoud L, Klemm U, Engel W. The lack of protamine 2 (P2) in boar and bull spermatozoa is due to mutations within the P2 gene. Nucleic Acids Res 1990;18:1249–54.
[294] Queralt R, Adroer R, Oliva R, Winkfein RJ, Retief JD, Dixon GH. Evolution of protamine P1 genes in mammals. J Mol Evol 1995;40:601–7.
[295] Steger K. Transcriptional and translational regulation of gene expression in haploid spermatids. Anat Embryol 1999;199:471–87.
[296] Steger K, Balhorn R. Sperm nuclear protamines: a checkpoint to control sperm chromatin quality. Anat Histol Embryol 2018;47:273–9.
[297] Shang E, Salazar G, Crowley TE, Wang X, Lopez RA, Wang X, et al. Identification of unique, differentiation stage-specific patterns of expression of the bromodomain-containing genes Brd2, Brd3, Brd4, and Brdt in the mouse testis. Gene Expr Patterns 2004;4:513–9.
[298] Berkovits BD, Wang L, Guarnieri P, Wolgemuth DJ. The testis-specific double bromodomain-containing protein BRDT forms a complex with multiple spliceosome components and is required for mRNA splicing and 3'-UTR truncation in round spermatids. Nucleic Acids Res 2012;40:7162–75.
[299] Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S, Khochbin S. Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. Mol Cell Biol 2003;23:5354–65.
[300] Govin J, Lestrat C, Caron C, Pivot-Pajot C, Rousseaux S, Khochbin S. Histone acetylation-mediated chromatin compaction during mouse spermatogenesis. Ernst Schering Res Found Workshop 2006:155–72.
[301] Dhar S, Thota A, Rao MR. Insights into role of bromodomain, testis-specific (Brdt) in acetylated histone H4-dependent chromatin remodeling in mammalian spermiogenesis. J Biol Chem 2012;287:6387–405.
[302] Manterola M, Brown TM, Oh MY, Garyn C, Gonzalez BJ, Wolgemuth DJ. BRDT is an essential epigenetic regulator for proper chromatin organization, silencing of sex chromosomes and crossover formation in male meiosis. PLoS Genet 2018;14:e1007209.
[303] Onoda M, Djakiew D. A 29,000 M(r) protein derived from round spermatids regulates Sertoli cell secretion. Mol Cell Endocrinol 1993;93:53–61.
[304] Hodzic DM, Yeater DB, Bengtsson L, Otto H, Stahl PD. Sun2 is a novel mammalian inner nuclear membrane protein. J Biol Chem 2004;279:25805–12.
[305] Liu Q, Pante N, Misteli T, Elsagga M, Crisp M, Hodzic D, et al. Functional association of Sun1 with nuclear pore complexes. J Cell Biol 2007;178:785–98.
[306] Thoma H, Grunewald L, Braune S, Pasch E, Alsheimer M. SUN4 is a spermatid type II inner nuclear membrane protein that forms heteromeric assemblies with SUN3 and interacts with lamin B3. J Cell Sci 2023;136.
[307] Frohnert C, Schweizer S, Hoyer-Fender S. SPAG4L/SPAG4L-2 are testis-specific SUN domain proteins restricted to the apical nuclear envelope of round spermatids facing the acrosome. Mol Hum Reprod 2011;17:207–18.
[308] Huang SL, Chou TC, Lin TH, Tsai MS, Wang SH. Gcse, a novel germ-cell-specific gene, is differentially expressed during meiosis and gametogenesis. Reprod Sci 2013;20:1193–206.
[309] Morohoshi A, Miyata H, Oyama Y, Oura S, Noda T, Ikawa M. FAM71F1 binds to RAB2A and RAB2B and is essential for acrosome formation and male fertility in mice. Development 2021;148.
[310] Kaneko T, Toh S, Mochida I, Iwamori N, Inai T, Iida H. Identification of TMCO2 as an acrosome-associated protein during rat spermiogenesis. Mol Reprod Dev 2020;87:808–18.
[311] Liu W-S, Zhao Y, Lu C, Ning G, Ma Y, Diaz F, et al. A novel testis-specific protein, PRAMEY, is involved in spermatogenesis in cattle. Reproduction 2017;153:847–63.
[312] Endo S, Yoshitake H, Tsukamoto H, Matsuura H, Kato K, Sakuraba M, et al. TEX101, a glycoprotein essential for sperm fertility, is required for stable expression of Ly6k on testicular germ cells. Sci Rep 2016;6:23616.
[313] Burton J, Wojewodzic MW, Rounge TB, Haugen TB. A role of the TEX101 interactome in the common aetiology behind male subfertility and testicular germ cell tumor. Front Oncol 2022;12:892043.
[314] Kurita A, Takizawa T, Takayama T, Totsukawa K, Matsubara S, Shibahara H, et al. Identification, cloning, and initial characterization of a novel mouse testicular germ cell-specific antigen. Biol Reprod 2001;64:935–45.
[315] Takayama T, Mishima T, Mori M, Jin H, Tsukamoto H, Takahashi K, et al. Sexually dimorphic expression of the novel germ cell antigen TEX101 during mouse gonad development. Biol Reprod 2005;72:1315–23.
[316] Takayama T, Mishima T, Mori M, Ishikawa T, Takizawa T, Goto T, et al. TEX101 is shed from the surface of sperm located in the caput epididymidis of the mouse. Zygote 2005;13:325–33.
[317] Yin L, Chung CM, Huo R, Liu H, Zhou C, Xu W, et al. A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. Cell Mol Life Sci 2009;66:900–8.
[318] Feige E, Chen A, Motro B. Nurit, a novel leucine-zipper protein, expressed uniquely in the spermatid flower-like structure. Mech Dev 2002;117:369–77.
[319] Tesarik J. Fertilization of oocytes by injecting spermatozoa, spermatids and spermatocytes. Rev Reprod 1996;1:149–52.
[320] Vanderzwalmen P, Nijs M, Stecher A, Zech H, Bertin G, Lejeune B, et al. Is there a future for spermatid injections? Hum Reprod 1998;13(Suppl 4):71–84.
[321] Levran D, Nahum H, Farhi J, Weissman A. Poor outcome with round spermatid injection in azoospermic patients with maturation arrest. Fertil Steril 2000;74:443–9.
[322] Ogonuki N, Inoue K, Ogura A. Birth of normal mice following round spermatid injection without artificial oocyte activation. J Reprod Dev 2011;57:534–8.
[323] Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 1995;121:2397–405.
[324] Lei ZL, Huang JC, Shi LH, Miao YL, Nan CL, Ouyang YC, et al. Development of normal mice after microinjection of round spermatids into oocytes stored at room temperature for one day. Mol Reprod Dev 2008;75:795–800.
[325] Ock SA, Lee SL, Jeon BG, Cho SR, Kumar BM, Choi YS, et al. Isolation and viability of presumptive spermatids collected from bull testes by Percoll density gradient. Anim Reprod Sci 2006;93:144–56.
[326] Lee JW, Kim NH, Lee HT, Chung KS. Microtubule and chromatin organization during the first cell-cycle following intracytoplasmic injection of round spermatid into porcine oocytes. Mol Reprod Dev 1998;50:221–8.
[327] Yanagida K, Katayose H, Yazawa H, Kimura Y, Sato A, Yanagimachi H, et al. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum Reprod 1999;14:1307–11.
[328] Sofikitis NV, Toda T, Miyagawa I, Zavos PM, Pasyianos P, Mastelou E. Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. Fertil Steril 1996;65:176–85.
[329] Choi JY, Lee EY, Cheong HT, Yoon BK, Bae DS, Choi DS. Effects of activation timing on the fertilization rate and early embryo development in porcine ROSI procedure. J Assist Reprod Genet 2004;21:329–34.
[330] Liu XY, Miao YL, Zhang J, Qiu JH, Cui XZ, Gao WQ, et al. Effects of activation on functional aster formation, microtubule assembly, and blastocyst development of goat oocytes injected with round spermatids. Cell Reprogr 2012;14:436–47.
[331] Sakamoto M, Ito D, Inoue R, Wakayama S, Kikuchi Y, Yang L, et al. Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection. Development 2022;149.
[332] Wang J, Zhou C, Gao S, Song X, Yang X, Fan J, et al. Single-cell multiomics sequencing reveals the reprogramming defects in embryos generated by round spermatid injection. Sci Adv 2022;8:eabm3976.
[333] Soto-Heras S, Roura M, Catalá MG, Menéndez-Blanco I, Izquierdo D, Fouladi-Nashta AA, et al. Beneficial effects of melatonin on in vitro embryo production from juvenile goat oocytes. Reprod Fertil Dev 2018;30:253–61.

CHAPTER 2: Aim of the thesis

This thesis aims to develop biotechnological strategies to shorten the generational interval in cattle (*Bos taurus*), with the ultimate goal of accelerating genetic selection for animals that are more resilient to climate change and have a reduced environmental footprint. We propose deriving male gametes from genetically superior embryos to fertilize mature oocytes, thereby generating a cohort of embryos with high genetic merit. Iterating this cycle multiple times could significantly shorten generation intervals and accelerate genetic progress. However this process remains unachieved in large mammals.

Given the intricacy of the full procedure, this Ph.D. project focused on three foundational steps:

1. **Isolation and characterization of bovine spermatids**, followed by assessment of their fertilizing potential through intracytoplasmic spermatid injection (ICSI) into *in in vitro*–matured oocytes.

2. **Development of a three-dimensional testicular bioscaffold**, designed to replicate the native tubular architecture and physiological microenvironment of the testis, thereby providing a supportive niche for meiotic progression.

3. **Isolation and characterization of the main functional testicular cell types**, Sertoli cells and spermatogonia, using specific markers.

CHAPTER 3: Isolation and characterization of bovine spermatids and evaluation of their fertilizing ability in vitro

Aim of the chapter

Given that the final objective is to produce offspring using male gametes derived directly from embryonic cells, we chose to focus on the generation of spermatids rather than fully mature spermatozoa, as the latter would introduce an additional layer of complexity. In murine models, spermatids have successfully supported embryonic development when injected into oocytes via intracytoplasmic injection, resulting in viable offspring. These findings demonstrate that, under specific conditions, spermatids possess the developmental competence required for fertilization.

However, the expected low efficiency of *in vitro* gametogenesis from somatic cells, combined with the difficulty of replicating the precise epigenetic reprogramming events necessary for normal gamete differentiation, presents significant challenges for adapting these protocols to livestock species. Therefore, we initiated the development of a procedure for isolating bovine spermatids from adult and prepubertal animals and assessing their fertilizing capacity through intracytoplasmic injection into *in vitro* – matured oocytes.

Additional limitations to the application of this technology in cattle include the complex architecture of the testis and the lack of specific

molecular markers that can reliably distinguish spermatid stages in this species. The work presented in this chapter focuses on establishing a reliable and reproducible protocol for the isolation, identification, and quality assessment of bovine spermatids, as well as evaluating their fertilizing potential *in vitro*.

The initial phase involved mechanical and enzymatic dissociation of testicular tissue to obtain a heterogeneous cell suspension, followed by gradient centrifugation to enrich the spermatid population. Identification of the correct germ cell stage was based on morphological evaluation via light microscopy, supported by molecular analyses using established markers of meiotic and post-meiotic differentiation. Isolated spermatids were then subjected to viability testing, DNA integrity assays, and ploidy analysis to assess their biological quality. Selected spermatids were injected into *in vitro*–matured bovine oocytes to evaluate their fertilizing ability.

Overall, the results indicate that fertilization efficiency remains lower than that achieved with conventional intracytoplasmic sperm injection (ICSI), confirming the limited competence of spermatids to fertilize mature oocytes. Despite being haploid and theoretically capable of fertilization, spermatids, in fact, lack several structural and functional features of

mature spermatozoa, including motility, nuclear condensation, and the ability to activate the oocyte.

The details of this set of experiments are described in the article entitled *"Development of a procedure for isolation, identification and quality assessment of bovine spermatids and evaluation of their fertilizing ability in vitro"*, published in *Frontiers in Bioengineering and Biotechnology* (DOI: 10.3389/fbioe.2025.1581019), presented here in its original published form.

Since the publication of our manuscript, a recent study has reported the generation of bovine and sheep offspring from spermatid-like haploid cells (Yang et al., 2025). However, in that study, the spermatids were derived from genetically manipulated haploid embryonic stem cell lines. Specifically, the loss of asymmetrical histone modifications in the pronuclei following intracytoplasmic injection of haploid ES cells was a major barrier to embryo development. This was overcome by exogenous expression of protamine in ruminant haploid ES cells. While technically impressive, this highly complex procedure diverges from our primary objective: the rapid generation of a spermatid from a specific embryo of high genetic merit, rather than from a cell line.

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Development of a procedure for isolation, identification and quality assessment of bovine spermatids and evaluation of their fertilizing ability *in vitro*

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Intracytoplasmic spermatid injection into oocytes has limited efficiency in cattle, with no offspring generated so far, partly due to ambiguous spermatid identification. This study aimed to develop and validate a method for isolating and characterizing bovine spermatids to improve the efficiency of spermatid intracytoplasmic injection. First, we optimized a protocol for spermatid isolation from bull testis using a discontinuous Percoll gradient and 10 µm mesh cell strainers. Next, we established a stage-specific separation strategy based on DNA content, size, and granularity using flow cytometry to distinguish round and elongating/elongated spermatids suitable for molecular analysis. Morphological assessment confirmed that 72.5% of isolated cells were at the spermatid stage, supported by a high haploidy rate, spermatid-specific transcript expression (*PRM1*, *PRM2*, *SPACA9*, *SPERT*), and SPERT protein detection. Viability assays showed that spermatids maintained intact DNA at 0 and 24 h at 4°C and 37°C, though mitochondrial activity and ROS levels increased over time, suggesting oxidative stress. When spermatids were injected into oocytes (n = 82), only 13.4% formed two pronuclei, whereas 46.3% exhibited a single pronucleus and a condensed chromatin spot, indicating incomplete activation or fertilization failure. This work contributes to refining bovine intracytoplasmic injection protocols. Future applications of this approach, particularly if functional spermatids can be derived from spermatogonia or embryonic cells, could help shorten the generational interval in cattle breeding.

KEYWORDS

cattle, bull testis, spermatids, intracytoplasmic spermatid injection, flow cytometry cell sorting, embryotechnology

1 Introduction

Modern agricultural advancements have transformed livestock production, increasing the need for efficient breeding strategies (Ritchie and Roser, 2024). In cattle, optimizing genetic selection is crucial to meet the rising global demand for food (Henchion et al., 2021), while improving disease resistance, climate resilience, and animal welfare (Berglund, 2015). One possible approach to enhance breeding efficiency is by shortening the generation interval, which can accelerate genetic improvement and adaptability (Pasquariello et al., 2024).

Genomic selection (GS) has been widely adopted in livestock breeding programs to accelerate genetic gain, boosting production efficiency and promoting the sustainability of animal agriculture. When GS is combined with Assisted Reproductive Techniques (ARTs), also known as embryotechnology, the highest increase in genetic gain is achieved by reducing the generation interval (Cenariu et al., 2012). Multiple Ovulation Embryo Transfer (MOET), also known as *in vitro* derived embryos (IVD) (Reuben and Bó, 2020), allows increased female selective pressure by reducing the generation interval to approximately 1 year (Lafontaine et al., 2023). The emerging frontiers of generating functional male and female gametes *in vitro* from neonatal gonads or even embryonic cells are paving the way for an innovative approach that could shorten the generational interval to a few weeks (Hayashi et al., 2012; Goszczynski et al., 2019). This strategy involves using immature cells, such as round and elongated spermatids, for intracytoplasmic injection into *in vitro* matured (IVM) oocytes. Spermatid injection has been successfully used in human (Tanaka et al., 2015) and mouse (Zhu et al., 2024), leading to full-term offspring. However, its use in cattle is still limited due to difficulties in identifying, retrieving and handling spermatids, and low fertilization and embryo development rates (Ock et al., 2006a; 2006b).

This work aimed to develop a protocol for isolating bovine spermatids from bull testis and to characterize their morphological features for accurate identification. Additionally, we aimed to confirm spermatid haploidy, assess the expression of key spermatid markers, and evaluate their viability and quality under different culture conditions. Furthermore, we sought to assess the fertilizing ability of isolated spermatids and establish a flow cytometry procedure for stage-specific spermatid isolation based on DNA content, size, and granularity.

2 Materials and methods

All reagents were purchased from Sigma-Aldrich, Milan, Italy, unless otherwise indicated. If not stated, each experiment was performed in 5 replicates.

2.1 Ethical statement

Bovine testes were collected from an authorized local slaughterhouse. This study did not involve the use of living animals; therefore, ethical approval was not required.

2.2 Spermatid isolation protocol

Testes were collected from 10 mature Charolaise bulls (24 months of age), destined for meat production, immediately after slaughtering. Testis were transported on ice to the laboratory within 1 h (h) to ensure optimal tissue preservation. After testis decapsulation, testicular parenchyma was washed twice with Dulbecco's phosphate-buffered saline (DPBS). Thereafter, 10 mg of tissue was minced into small pieces with scalpels. Tissue pieces were then incubated into 20 mL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Thermofisher) containing 0.2 mg/mL Pronase at 37°C for 3 min. To stop enzyme activity, 10 mL DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) were added to the digested tissue pieces. Thereafter, cell clumps and debris and obtained cell suspension were filtered using 40 and 20 µm nylon mesh cell strainers. Then, the tube was centrifuged at 500 × g for 5 min (min) to precipitate the cells. The cell pellet was washed twice with DPBS followed each time by centrifugation at 500 × g for 5 min. The cell pellet was then resuspended using 0.5 mL DMEM/F12 and layered on a discontinuous Percoll gradient made of 2 mL each of 90%, 45%, 40%, 35% and 20% Percoll solutions diluted with DMEM/F12 using a 15 mL conical tube as described in previous work (Ock et al., 2006b; 2006a). The tube was centrifuged for 25 min at 650 x g. After centrifugation, 35% and 40% Percoll gradients were collected and washed three times using 5 mL DPBS and once using 1 mL DMEM/F12 supplemented with 10% FBS. Cell suspension was then filtered using a 10 µm nylon mesh cell strainer. After isolation, cells were immediately analysed or used for round spermatid injection. In some experiments, their quality was assessed after 24 h of culture. An overview of the isolation procedure is shown in Figure 1.

2.3 Spermatid identification and characterization

2.3.1 Morphological evaluation and identification

Spermatids were identified by morphological assessment upon staining with Haematoxylin and Eosin or 2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI) (Thermofisher). To this end, cells were washed 2–3 times using 2 mM Ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) and diluted in 0.2 mL PBS 1% BSA containing 30,000–50,000 cells and spin onto slides for 5 min at 112 x g. The obtained slides were air dried for 10 min, fixed using PFA 4% and stored ad 4°C in PBS.

2.3.2 Assessment of haploidy using FISH analysis

Isolated spermatids were washed twice in 6 mM EDTA PBS and fixed using 3:1 methanol: acetic acid solution. A 5 µL droplet of fixed cell suspension was dropped on a clean microscope slide and air dried at room temperature. DNA was denaturation was performed by submerging each slide in 3 M sodium hydroxide at room temperature for 2 min, facilitating DNA decondensation. Thereafter, the slides were washed in distilled water, dehydrated by sequential submersion in 70, 80, and 96% ethanol solutions (for

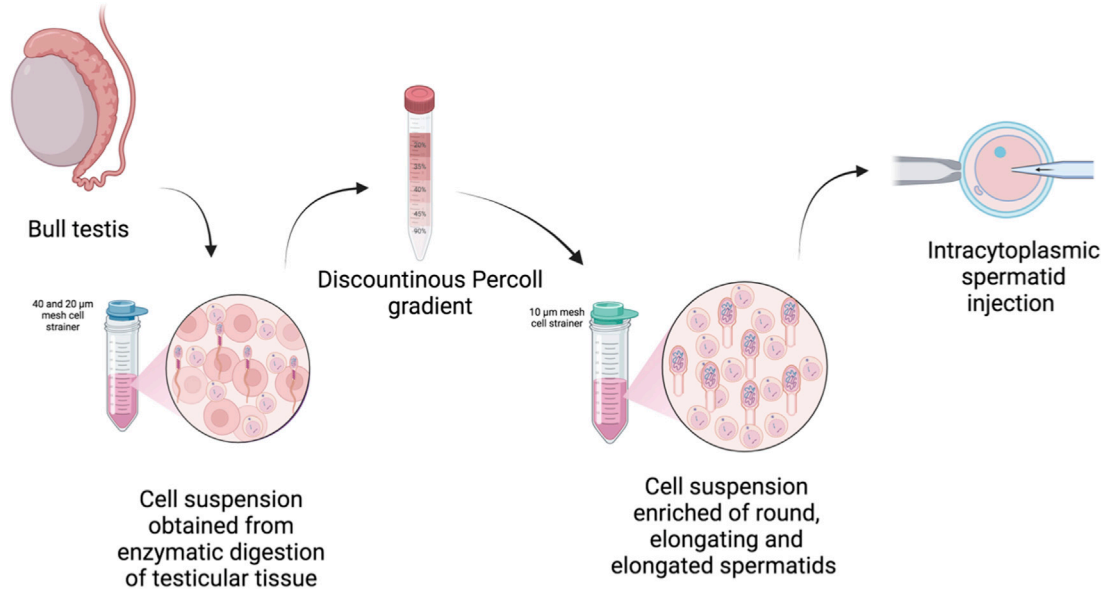

FIGURE 1
Overview of the procedure used to isolate bovine spermatids. Created in BioRender: https://BioRender.com/3rs70m1.

TABLE 1 Bacterial artificial chromosome (BAC) probes with their genome view (according to NCBI) used for dual colour FISH analysis. For each probe, total length in base pairs (bp), genome localization, label and imposed color are reported.

Probe	Total length (bp)	Bos taurus UMD 3.1.1 Genome localization (bp)	Label	Imposed color
1st pool				
bI 0004H06	83.78	22,452,603–22,536,386	Digoxigenin	Red
bI 0696G12	122.39	23,090,214–23,212,604	Digoxigenin	Red
bI 0042A07	242.34	23,287,629–23,529,972	Digoxigenin	Red
2st pool				
bI 0039B11	100.79	44,410,860–44,511,655	Biotin	Green
bI 0393A06	147.11	43,921,537–44,068,649	Biotin	Green
bI 0651C08	102,98	44,560,941–44,663,929	Biotin	Green

2 min each), and air-dried before hybridization following the protocols described by Iannuzzi et al. (2023). The probes used in the study consisted of pools of three Bacterial Artificial Chromosomes (BACs), selected as a contig to cover approximately 1 megabases of sequence, enhancing FISH signal intensity (Table 1). Probe preparation and dual-color FISH analysis were carried out according to the protocols described of Genualdo et al. (2021), with slight modifications. Briefly, probes were labeled using the Biotin and Dig-Nick Translation Mix kit (Roche Applied Science) as detailed in Table 1. They were denatured at 70°C for 10 min, then pre-annealed at 37.0°C for 60.0 min. For each slide, two probes were simultaneously hybridized to the decondensed sperm heads, covered with 24-mm coverslips, sealed, and incubated at 37°C in the humidified chamber for 24 h. Post-hybridization washes included two 5-minute incubations in 50% formamide in 2X saline-sodium citrate (SSC) at 45°C, followed by two additional 5-minute washes in 2X SSC at 45°C, following the protocols described by Dio et al. (2020). Hybridization sites were detected using indirect labeling FITC-avidin for the biotinylated probes and an anti-digoxigenin antibody conjugated to a red fluorophore for the digoxigenin-labeled probes. Slides were incubated for 1 h in the dark, humid chamber at 37°C for 1 h. After staining, they were counterstained with Vectashield DAPI H-1000 antifade solution (Vector Laboratories).

Slides were analyzed using a Leica DM 5500B fluorescence microscope equipped with a 100X objective, specific triple-bandpass filters (DAPI, FITC, Orange), and a high-sensitivity monochrome camera. Digital images were acquired in grayscale, and false-color images were generated using Cytovision-Leica software to differentiate signal types. Haploid spermatids were identified when a single signal was observed for each probe (Figure 2C).

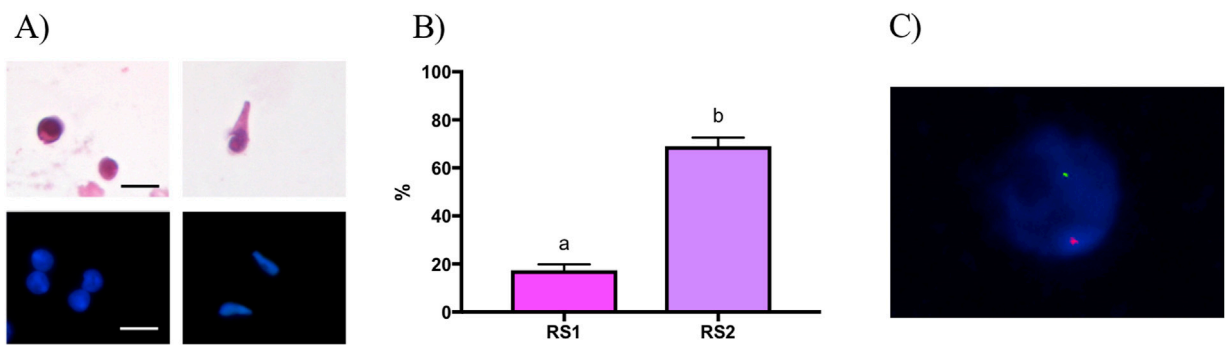

FIGURE 2
Identification of spermatids by morphological and haploidy evaluation. **(A)** Representative picture of isolated round and elongated spermatids stained with Hematoxylin and Eosin (top) and DAPI (bottom). **(B)** Results of morphological analysis performed to assess the percentage of spermatid after enzymatic digestion of testicular tissue (RS1) and after their isolation using the procedure described in Figure 1 (RS2). **(C)** Representative picture of nucleus stained using DAPI (blue) and FISH chromosome signals (red and green dots) for identification of haploid spermatids.

TABLE 2 List of primers used for real time quantitative PCR analysis. For each reverse and forward primer, the name ID of the gene. The accession number and the forward and reverse sequences are reported.

Name ID	Accession number ID	Forward primer (5′->3′)	Reverse primer (5′->3′)
PRM1	NM_174,156.2	CAGCCCACAAATTCCACCT	TGAGGCGCATCGGTATCT
PRM2	NM_174,157.4	CGCTACCACTACAGACACAG	AAGCTTAGAGCTGCCTTCC
SPERT	NM_152,719.3	CTGAAATGTAGGGTGGAGGAATC	GTACAAGTTGTGGAGCCTCAG
SPACA9	NM_001101193.2	GAACGCCCACGACAAGAT	GCAGATGTCCAGGAACATGA
GAPDH	NM_001034034.2	TCATCATCTCTGCACCTTCTG	ATGCCAAAGTGGTCATGGA
ACTB	NM_173,979.3	TCTTCCAGCCTTCCTTCCT	TAGAGGTCCTTGCGGATGT

2.3.3 Gene expression analysis

Gene expression analysis was carried out by reverse transcription quantitative real-time PCR (RT-qPCR) for the following genes (Table 2): protamine 1 (*PRM1*), protamine 2 (*PRM2*), Spermatid-associated protein (*SPERT*), sperm acrosome associated 9 (*SPACA9*). A total of three biological replicates for each group (RS1 and RS2) were used for RT-qPCR. RNA extraction was carried out using TRIZol (15596018, Thermofisher) reagent combined to PureLink RNA Mini Kit (12183018A, Thermofisher) with on column DNAse treatment using the PureLink DNase (12185010, Thermofisher). Complementary DNA (cDNA) was synthesized using iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, USA) following the manufacturer's protocol. The cDNA samples were diluted 1:3 using RNase-free water and stored at −20°C until RT-qPCR was run. Each PCR reaction was performed in triplicate using 10 µL SsoAdvanced™ Universal SYBR Green Supermix (1725271, BioRad), 1.2 µL 10 µM primer mix, 6.8 µL nuclease free water and 2 µL 1:3 diluted cDNA sample using 96 well plates (HSP9601, Bio-Rad). The RT-qPCR program was run on a Bio-Rad CFX96™ Real time machine (Bio-Rad), as follows: 95°C for 30 s cycle followed by 40 cycles of amplification step at 95°C for 15 s and 59°C for 30 s. A melting curve was analysed for each experiment to assess the specificity of primer amplification. Relative gene expression was calculated using the 2^{-ddCt} method (Pfaffl, 2001). Normalization of Ct values was obtained using the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta actin (*ACTB*).

2.3.4 Immunofluorescence of spermatid associated protein (SPERT)

After spinning cells onto the slides using cytospin following the procedure described above, they were immediately fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. Aspecific bindings were prevented by incubating cells in 10% goat serum in PBS for 30 min at room temperature. Afterwards, cells were stained using anti-SPERT primary antibody (1:150 dilution, ab243570, abcam) overnight at 4°C. Subsequently, cells were incubated with secondary antibody Alexa Fluor™ 594 goat anti-rabbit (1:250, A11012, Life Technologies Corporation) for 30 min at room temperature. Nuclei were counterstained with DAPI for 15 min at room temperature. Images were acquired using an Eclipse TE200 microscope (Nikon).

2.4 Assessment of spermatid quality during *in vitro* culture

2.4.1 *In vitro* culture of spermatids

Quality assessment was carried out on the cells collected immediately after isolation and after 24 h of culture. In details, spermatids were either stored at 4°C in air atmosphere or cultured in DMEM/F12 medium supplemented with 10% FBS at 37°C in 5%

CO_2. Cell quality was examined scoring cell viability (LIVE/DEAD staining), DNA integrity (acridine orange), and mitochondrial activity (red CMXRos and green FM mitoTrackers) and analysis of reactive oxygen species (Image-iT Live Green ROS).

2.4.2 DNA integrity

Isolated cells were smeared on polylysine slides, air dried and fixed in alcoholic solution (methanol CH_3OH and acetic acid CH3COOH 3:1) overnight. Acridine orange (10 mg/mL) solution pH 2.5 was prepared with 0.1 M of Citric Acid and 0.3 M of Na_2HPO_4. Slides were stained with acridine orange solution for 10 min and air-dried. Then cells were observed under a fluorescence microscope.

2.4.3 Cell viability

LIVE/DEAD™ Cell Imaging Kit (488/570) (R37601, Invitrogen) was used to determine the number of viable cells based on a sensitive two-color fluorescence cell viability assay optimized for evaluating intracellular esterase activity and plasma membrane integrity. Viability was quantified by dividing the number of live cells by the total number of cells and showed as a percentage of live and dead cells.

2.4.4 Determination of reactive oxygen species

ROS concentration in single spermatid was measured using the Image-iT Live Green ROS detection kit (Invitrogen). Briefly, cells were washed three times in PBS. Cells were then stained with 25 μM 5- and 6-carboxy-20,70- dichlorodihydrofluorescein diacetate (carboxy-H2-DCFDA) in the dark for 30 min. Subsequently, cells were washed three times and immediately imaged using an Eclipse TE200 microscope (Nikon) with excitation of cells at 480 nm and emission at 510 nm. Image analysis was carried out using Fiji ImageJ (Pasquariello et al., 2018), and ROS concentration was calculated and reported as relative fluorescence units.

2.5 Intracytoplasmic injection into mature oocytes

2.5.1 Oocyte collection and *in vitro* maturation (IVM)

Cumulus oocyte complexes (COCs) were isolated from ovaries, obtained from culled cows at a local slaughterhouse, by vacuum pump aspiration of 2–6 mm ovarian follicles, as previously described (Modina et al., 2007). Only COCs with at least 5 complete, compact cumulus layers and finely granulated ooplasm were selected for IVM culture in $NaHCO_3$-buffered TCM199 supplemented with 0.68 mM L-glutamine, 0.4% fatty acid-free bovine serum albumin, 0.2 mM sodium pyruvate and 0.1 international units/mL of recombinant human follicle-stimulating hormone (r-hFSH, Gonal-F, Merck Serono SpA) in humidified air under 5% CO_2 at 38.5°C for 22 h (Luciano et al., 2013). After 22 h, the cumulus cells were mechanically removed by gentle pipetting and cultured in the same IVM conditions for additional 2–4 h to allow recovery from the cumulus removal procedure and clearly visualize the extrusion of the first polar body (PBI).

2.5.2 Spermatid injection into mature oocytes

The isolated spermatids were centrifuged to remove excess supernatant. A 2 μL aliquot of the spermatid suspension was added to one of the 5–6 droplets of 20 μL Hepes-buffered Tyrode's albumin lactate pyruvate (TALP) on the microinjection dish. A 10 μL drop of 10% polyvinylpyrrolidone (PVP) (FertiCult) was placed at the center of the dish for priming the injection pipette, facilitating smooth injection. The dish was overlaid with OVOIL (Vitrolife), and the oocytes were transferred into the surrounding TALP droplets for injection. Spermatid injection was performed using a Nikon Ti2 Eclipse microscope equipped with a Narishige Takanome micromanipulator. Injection needles (Origio, Cooper Surgicals) with inner diameters (ID) 6.6–7.9 μm were used, along with holding needles (Kitazato) with an outer diameter (OD) of 120 μm. After loading the spermatids in the needle and orienting the oocyte with the polar body at 6 or 12 o'clock, the needle was inserted into the oocyte. The ooplasm was partially aspirated and released 2–3 times to ensure the oolemma was broken before depositing the spermatid into the cytoplasm. Oocytes were injected in batches of 10–15, and some were kept not injected as a control group.

2.5.3 Oocyte activation and culture

All the injected oocytes and the not injected control group underwent a dual activation protocol (Horiuchi et al., 2002; Abdalla et al., 2009). First, they were incubated with 5 μM calcium ionophore (A23187, Sigma-Aldrich) for 5 min and cultured for 4 h in IVF medium ($NaHCO_3$-buffered TALP) at 38.5°C, 5% CO_2 in humidified air. Then a second activation with 7% ethanol for 5 min was applied, followed by 19–24 h culture in synthetic oviductal fluid (SOF) supplemented with 5% FBS at 38.5°C, 5% CO_2, 5% O_2 in humidified air. Finally, prospective zygotes were fixed in 4% paraformaldehyde, stained with DAPI-containing mounting media (Vectashield), and analyzed for two-pronuclei formation.

2.6 Fluorescent-activated sorting of spermatid populations by flow cytometry

In a different set of experiment, we aimed to separate the haploid spermatid subpopulations based on apparent DNA content, size, and granularity. In particular, we adapted the protocol originally described in the mouse (Simard et al., 2015). Briefly, testicular cells used for fluorescent-activated cell sorting (FACS) were obtained by digesting the testicular parenchyma using the digestion medium made with DMEM/F12 and pronase as described in 2.2. The obtained cell suspension was passed through a 40 μm nylon mesh cell strainer and washed 2–3 times using PBS before being resuspended into 3 mL PBS containing 2 mM EDTA pH 7. To fix isolated cells, 9 mL (3 volumes) of ice-cold 100% ethanol were slowly added using a 10 mL serological pipette with gentle agitation using a vortex at low speed. Then they were incubated for 15 min on ice and mixed every 3 min by inversion. Fixed cells were washed 3 times using a sorting buffer prepared with PBS containing 2 mM EDTA and 5% Serum Bovine Albumin. Cells were precipitated each time by centrifugation at 500 x g for 8 min. FACS was performed using BD FacsMelody - Cell Sorter. Briefly, cells were resuspended in 1 mL sorting buffer and stained using 15 μM SYTO 16 (cat.no. S7578, Invitrogen) for 30 min in the dark at 4 °C. An aliquot of unstained

TABLE 3 Assessment haploidy cells using fluorescent *in situ* hybridization. For each bull, percentage of spermatids, diploid cells and no signal are reported.

Sample	Spermatids (n) (%)	Diploid cells (2n) (%)	No signal (%)
Bull 1	74.0	11.0	15.0
Bull 2	72.0	21.0	7.0
Bull 3	67.0	21.0	12.0

cells was kept separate to be used as a negative control. During cell sorting, the nozzle was set up to 100 μm, sort mode to 4 Way Purity with flow rate as 1.0 by which about 1,000 events per seconds were processed. Moreover, sample agitation was set up at 300 rpm and a temperature of 4 °C. Gating strategies were optimized to use a 488 nm laser-equipped cell sorter following the gating scheme detailed in Figure 6A and excluding small debris and cell doublets. Immediately after sorting, to carry out an epifluorescence microscopy evaluation of collected populations, collected cells were plated into 12-well plates, and nuclei were imaged using an Eclipse TE200 microscope (Nikon) with excitation at 480 nm and emission at 510 nm.

2.7 Statistical analyses

Before performing statistical analysis, data were checked for normality and homoscedasticity and it was confirmed that all the values were normally distributed. Subsequently, Fishers' chi-square exact test and one-way ANOVA (SPSS 19.1; 240 IBM), followed by *post hoc* Tukey's test, were performed depending on the number of experimental groups. All the results are expressed as the Mean ± Standard Error of the Mean (SEM), and differences in p-value ≤0.05 were considered statistically significant.

3 Results

3.1 Isolation of alive spermatids

3.1.1 Identification of spermatids by morphological evaluation and fluorescent *in situ* hybridization

Morphological analysis revealed that round and elongated spermatids ranged between 7.0–12.0 μm in size. In round spermatids, nuclei were centrally located in the cell and had 1-3 nucleoli (Figure 2A). In elongated spermatids, nuclei were darker, polarized and nucleoli were absent (Figure 2A). In addition, 20.5% ± 1.2 of the cells collected after tissue digestion belonged to spermatid subpopulations, including round, early elongated and elongated spermatids (Figure 2B). Whereas, after completing the isolation procedure described in Figure 1, this value increased to 72.5% ± 2.2 ($P < 0.05$) (Figure 2B). In line with these results, FISH analysis showed that the percentage of haploid cells was on average equal to 76.7% ± 3.7 with respect to diploid cells (Figure 2C; Table 3).

3.1.2 Evaluation of spermatid marker expression

All spermatid cell markers analyzed, i.e., *PRM1*, *PRM2*, *SPERT* and *SPACA9*, were expressed in the cells obtained after tissue digestion (RS1) and in those collected after completing the isolating procedure (RS2). However, the expression level of all the transcripts was higher ($P < 0.05$) in the cells characterized by an enrichment in spermatid subpopulations (RS2), compared to the cells obtained right after tissue dissociation, confirming the efficiency of the procedure to increase the purity of collected spermatids (Figure 3A). In line with these results, SPERT protein expression was confirmed by immunofluorescence showing a specific cytoplasmic signal positivity in the spermatids (Figure 3B).

3.2 Culture and quality assessment of isolated spermatids

3.2.1 Evaluation of viability and DNA fragmentation

At 0 h, all cells were alive and had intact DNA (Figures 4A,B). At 24 h after culture, the percentage of alive cells was 98.2% ± 1.8% at 37°C and 97.8% ± 2.8% at 4°C ($P > 0.05$), respectively (Figure 4A, left). In agreement with these results, the percentage of cells with intact DNA was 99.0% ± 1.0% at 37°C and 98.5% ± 1.5% at 4°C ($P > 0.05$) (Figure 4A, right).

3.2.2 Mitochondrial activity evaluation and ROS quantification

Mitochondrial activity significantly increased ($P < 0.05$) in the spermatids cultured for 24 h at 37°C and 4°C compared to those analyzed at 0 h (Figure 4B, left). We hypothesized that oxidative stress was occurring during *in vitro* culture of spermatids. In line with our hypothesis, ROS concentration was higher ($P < 0.05$) in the spermatids cultured for 24 h at 37°C and 4°C compared to those analyzed at 0 h (Figure 4B, right).

3.3 Spermatid fertilizing ability

Figure 5A and supplemental video show the spermatid retrieval and oocyte injection. This procedure was carried out on 82 mature oocytes to assess the spermatid fertilizing ability. As graphically represented in Figures 5B,C, only 11 oocytes formed 2 PN upon activation (13.4%), while 38 displayed 1 PN (46.3%). Among the latter, 19 also showed a compact DNA spot within the cytoplasm that has been interpreted as the uncondensed spermatid. Notably, also non injected oocytes were sometimes able to form 2 PN, but at a much lower rate (1/33, approx. 3.0%), indicating that the majority of the 2 PN observed in the spermatid-injected group were bi-parental zygotes. The remaining oocytes were found either at the MII stage (n = 11), or at different/not interpretable stages (n = 16) or degenerated (n = 6).

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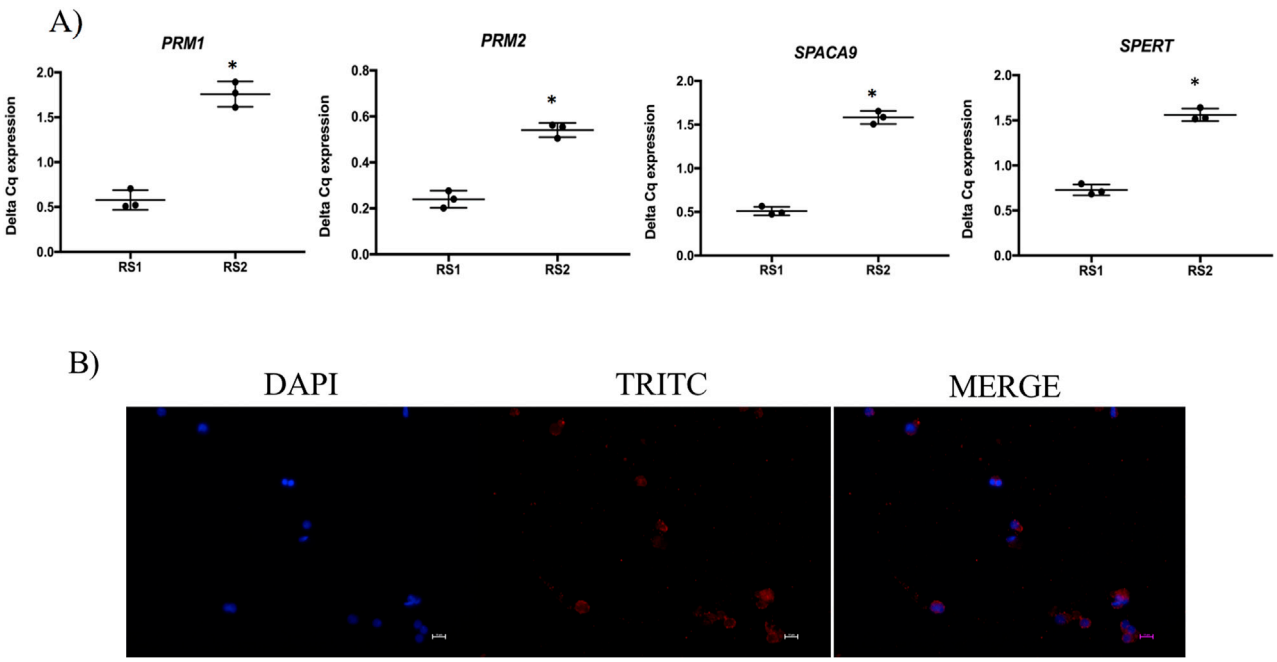

FIGURE 3
Gene and protein expression of spermatids. **(A)** Gene expression analysis of spermatid markers *PRM1*, *PRM2*, *SPACA9* and *SPERT* in the cells collected after enzymatic digestion of testicular parenchyma (RS1) and after carrying out the isolation procedure for purifying spermatids (RS2). **(B)** Representative immunofluorence microphotographs showing spermatid associated protein (SPERT) in the isolated spermatids, scale bar = 10 μm.

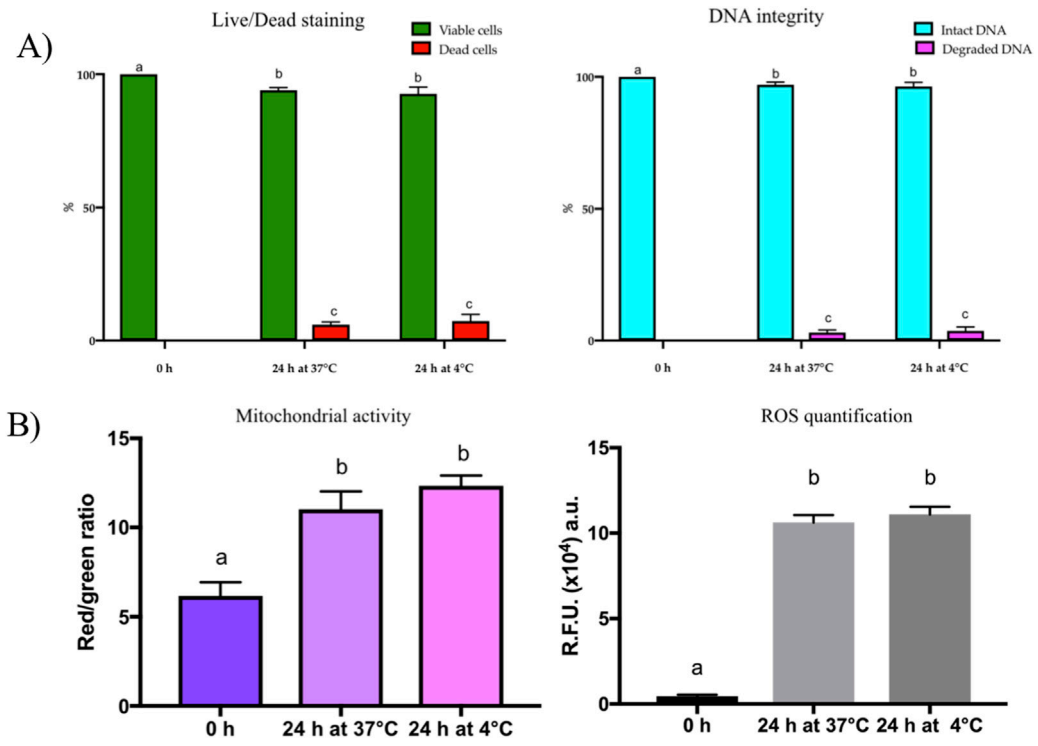

FIGURE 4
(A) Results of the viability (right) and DNA integrity (left) assays performed using LIVE/DEAD and acridine orange staining, respectively. Both assays were carried out immediately after round spermatid isolation (0 h) and after 24 h of culture at 37°C in 5% CO2 incubators (24 h at 37°C) and 4°C in air atmosphere (24 h at 4°C). **(B)** Results of mitochondrial activity (right) calculated as Red/green ratio and ROS quantification (left) immediately after round spermatid isolation (0 h) and after 24 h of culture at 37°C in 5% CO2 incubators (24 h at 37°C) and 4°C in air atmosphere (24 h at 4°C).

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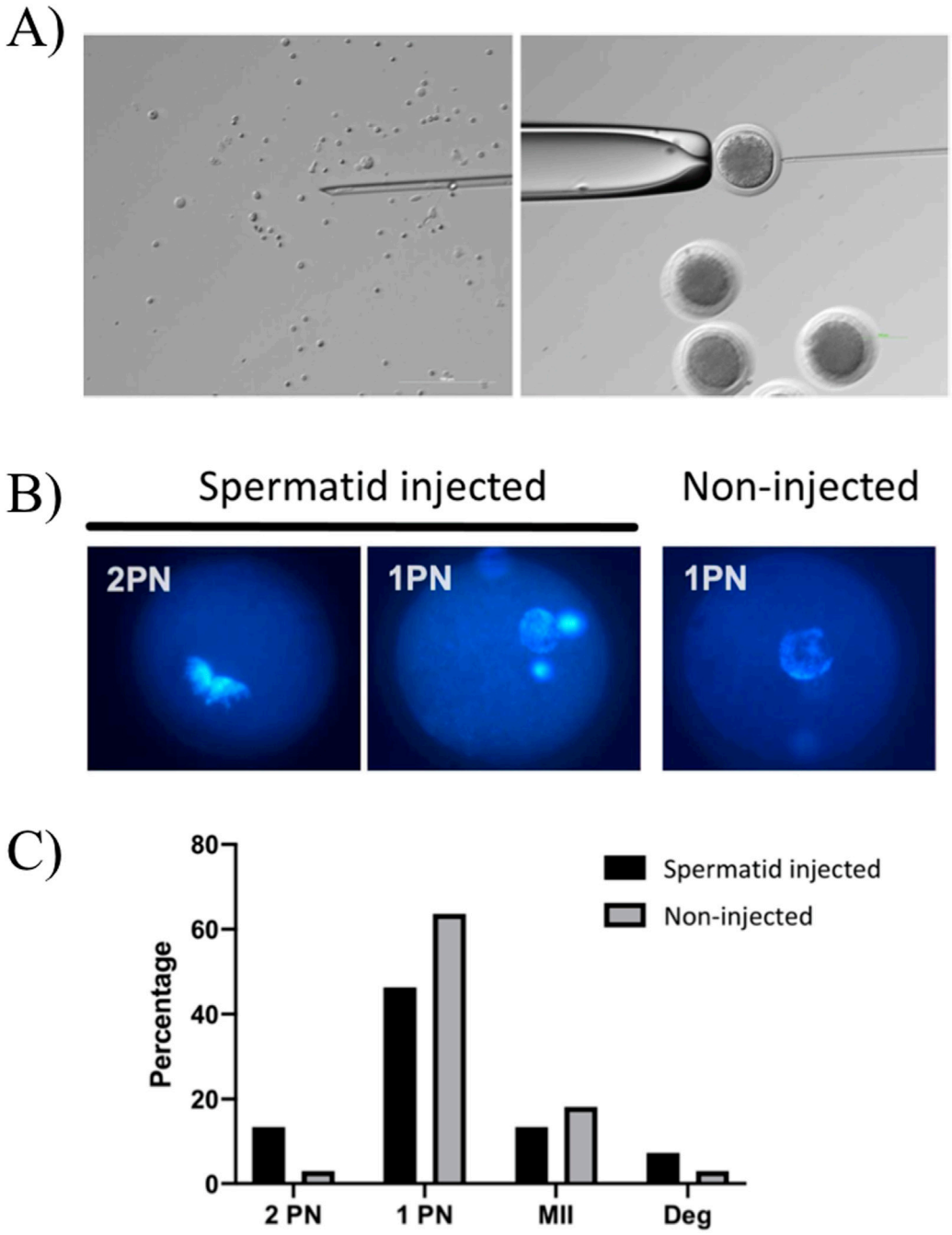

FIGURE 5
Spermatid injection in mature oocytes. **(A)** Representative images in bright field of spermatids ready to be injected (left) and injection in a mature oocyte (right). **(B)** Representative fluorescence images of zygotes with 2PN and 1PN in spermatid-injected and control, not injected oocytes. **(C)** Distribution of DNA configuration in spermatid-injected and control, not injected oocytes. Fishers' chi-square exact test, no significant difference.

3.4 Stage-specific spermatid isolation using flow cytometry

Cells positive for DNA staining were selected in the Gate 1. Thereafter, spermatids belonging to the spermatogenesis stages 1–12 were selected based on a dot plot showing granularity (SSC-A) *v*versus size (FSC-A) from gate 1. Then, spermatids from steps 1-9 and steps 10–12 were separated from each other according to the variation of DNA staining intensity (Gates 5 and 6). Spermatids from steps 13–14 and 15–16 were then selected from positively stained cells on a dot plot showing size (FSC-A) versus DNA staining (Alexa Fluor 488-A) represented by Gates 3 and 4. All sorted subpopulations were defined again with SYTO 16 FITC-W versus SYTO 16 FITC-A dot blots to boost their purity.

Epifluorescence microscopy evaluation revealed that steps 1-9 were represented by a homogenous cell population of round spermatids, characterized by a round nucleus and the presence of nucleoli (Figures 6B,C, right). Whereas steps 15–16 were

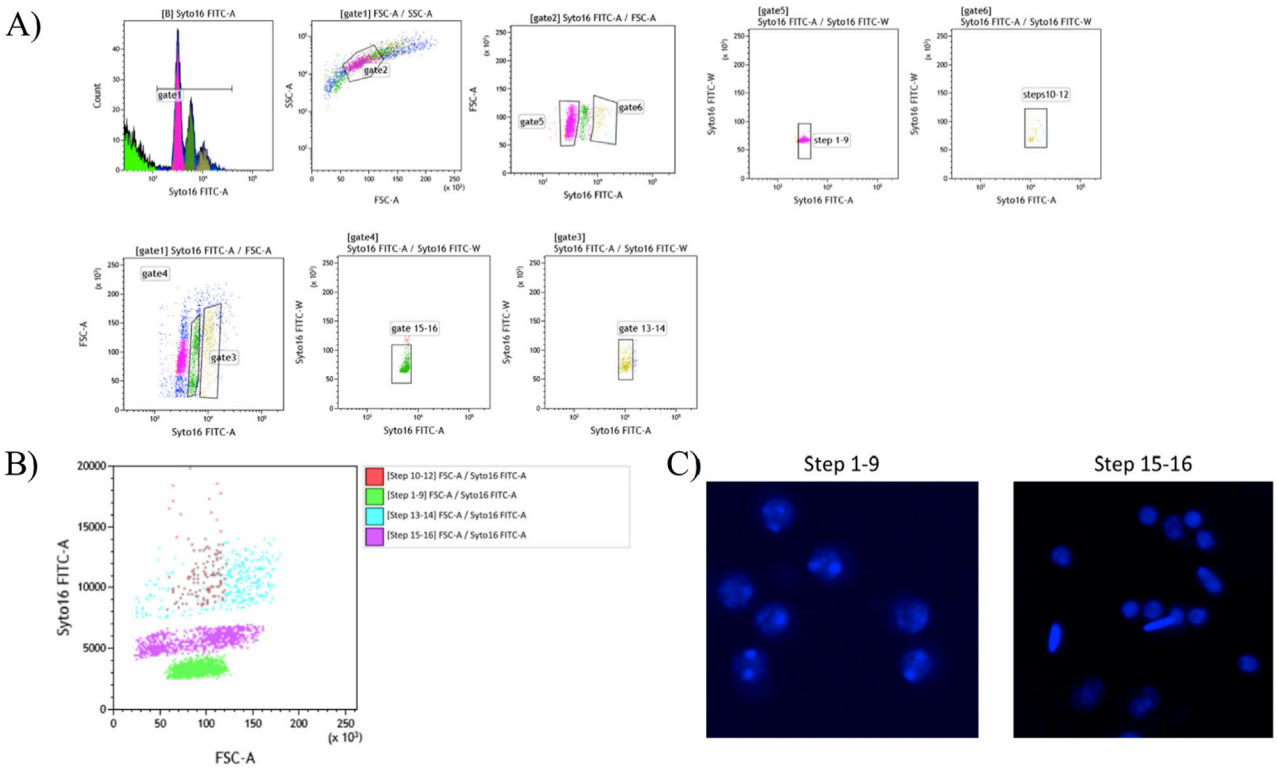

FIGURE 6
Flow cytometry approach for stage specific sorting of spermatid stage. **(A)** Gating strategy used with BD FacsMelody - Cell Sorter to sort pure spermatid subpopulations. **(B)** Flow cytometry data plots representing the steps used to isolate the spermatid subpopulations. **(C)** Representative microphotographs of steps 1–9 (right) and 15–16 (left) of the purified spermatid subpopulations.

represented by an heterogenous cell population of elongating and elongated spermatids with small and elongated nuclei (Figures 6B,C, left). In contrast, in steps 10–12 and 13–14, spermatids were not efficiently separated (Figure 6B).

4 Discussion

In the present work, we developed a protocol for isolating bovine spermatids, achieving a significant enrichment of spermatid subpopulations in the collected cells. In line with these results, isolated cells showed high haploidy rate and transcript levels of spermatid markers (*PRM1*, *PRM2*, *SPACA9* and *SPERT*). Immunocytochemistry confirmed SPERT protein expression specifically in the cytoplasm of spermatid stages. Cells remained viable with intact DNA at 0 and 24 h of culture at 37°C and 4°C. However, cultured cells were characterized by increased mitochondrial activity and ROS levels. In addition, spermatids showed a low fertilizing ability after injection into *in vitro* matured oocytes. Notably, for the first time, we established a protocol for stage-specific isolation of bovine spermatids using a flow cytometry approach.

In the first phase of the isolation procedure, spermatids represented only 20.5% of the cells obtained from tissue digestion. At the end of the procedure, their percentage increased to 72.5%, indicating the method's efficiency in selectively enriching for different spermatid stages. The methods for isolating spermatids have been mainly developed in mouse and include velocity sedimentation, density gradient (Bellve et al., 1977; Willison et al., 1990) centrifugal elution (Meistrich, 1977; Blanchard et al., 1991) immunoselection panning technique (Pelengaris and Moore, 1995) and BSA gradients (Kim et al., 2021; Li et al., 2024). In cattle, the use of Percoll density gradient resulted in only a total of 30%–40% of spermatids on the total cells isolated (Ock et al., 2006a; 2006b). Although all these procedures are not invasive for the cells, they are laborious and time-consuming. Here, we established a fast and easy-to-follow protocol, obtaining an efficient isolation of spermatids ready in only 1 hour that can be used for various other experiments. In addition, the morphological analysis confirmed the characteristics and composition of isolated spermatid subpopulations. Round and elongating/elongated spermatids were found to vary in size, ranging from 7.0 to 12.0 μm. In round spermatids, nuclei were centrally located with 1-3 nucleoli, while elongated spermatids exhibited darker, smaller or polarized nuclei lacking nucleoli. This distinction in nuclear and cell morphology aligns with the differentiation stages of spermatogenesis described in all mammalian species, including cattle (Staub and Johnson, 2018) and mice (Xu et al., 2021).

Supporting our results, FISH analysis further revealed that haploid cells were on average of a 76.7% of the total cell population isolated, once again confirming the efficiency of the procedure. These results also indicate the validity of the morphological identification approach that we performed to recognize all spermatid stages. In addition, these results were

supported by the higher transcript level of all the spermatid markers analyzed, *PRM1* (Steger, 1999), *PRM2* (Hamilton et al., 2019), *SPERT* (Steger, 1999) and *SPACA9* (Chen et al., 2008), in the cells collected with our isolation protocol. Interestingly, SPERT protein immunopositivity was observed in our purified cells. We think this is an interesting aspect since SPERT was recently described to be distinctive of the elongation stages of the spermatids but absent in mature spermatozoa, in primates, pigs and rodents (Feige et al., 2002). Our results, detecting SPERT protein in the cytoplasm of isolated bovine spermatids, demonstrate that this protein is conserved among mammalian species and could, therefore, be used as a distinctive marker of these cell type in cattle as well.

The evaluation of spermatid quality using viability and DNA integrity assays revealed that, at 0 h, all cells were alive with intact plasma membrane and DNA. After 24 h of culture, viability remained high with few cells dead at both temperatures (37°C: 98.2% ± 1.8%; 4°C: 97.8% ± 2.8%). These values were closely mirrored by the proportion of cells with intact DNA (37°C: 99.0% ± 1.0%; 4°C: 98.5% ± 1.5%). Taken together, *in vitro* culture conditions used were able to preserve spermatids' health as well as their genomic integrity. While we were expecting these results for cells cultured at 37°C, which is the standard temperature used for culturing mammalian cells, the results obtained from cells maintained at 4°C are intriguing. Our results are in agreement with (Martins et al., 2015) who described efficient spermatid preservation using refrigeration at 4°C. This suggests that spermatids can be preserved for 24 h, or even shipped to other laboratories, within this time frame before intracytoplasmic injection into mature oocytes.

It must be noted that culture spermatids showed significantly increased mitochondrial activity after 24 h compared to the 0-hour time point, regardless to the temperature. In the male reproductive system, mitochondria play a crucial role in developing and supporting germ cells, which are essential for producing healthy sperm (Costa et al., 2023). Several studies have shown that, *in vivo*, spermatogenic cells, including spermatids, require high consumption of lactate and glucose, metabolized through glycolytic and oxidative metabolism (Jutte et al., 1981; Bajpai et al., 1998). These molecules can enter the seminiferous tubules through Sertoli cells (Hall and Mita, 1984). Therefore, our findings may suggest that enhanced cellular metabolism during *in vitro* culture may result from tissue dissociation, which disrupts the interaction between Sertoli cells and spermatids. On the other hand, the described increase in metabolic activity is paralleled by an increase in ROS production in spermatids after 24 h, indicating the occurrence of oxidative stress. While mitochondrial activity is essential for cellular energy production, it is clear that excessive ROS levels can affect cell viability and function, potentially leading to oxidative damage over prolonged culture periods (Halliwell, 2003; Murphy et al., 2022). This suggests that further optimization of *in vitro* culture conditions may be beneficial to prevent oxidative stress and ensure long-term viability and function in the cultured spermatids. Future studies are mandatory to explore the use of antioxidants and/or other protective measures aimed at mitigating ROS accumulation while maintaining high viability and DNA integrity during extended culture time of spermatids.

Based on the results described above, we decided to perform intracytoplasmic spermatid injection into mature oocytes using spermatids immediately after their isolation.

The results of spermatid injection showed a rather low fertilizing ability, as only 13.4% of the mature oocytes formed 2 PN upon activation. Since the majority of the spermatid-injected oocytes went on to form 1 PN and few remained arrested at the MII, a possible interpretation is that the activation protocol is adequate to induce the maternal pronucleus formation. At the same time, spermatids fail to decondense in most of the cases. In line with this hypothesis, we often observed a spot of condensed DNA in the ooplasm of spermatid-injected oocytes along with 1 PN. We hypothesize that this DNA might derive from the injected spermatid, also because such a structure was absent in the non-injected, activated oocytes. Although confirmation using maternal and paternal pronuclei markers is still required, these initial results are encouraging, as they provide proof of concept that some bovine spermatids have acquired a certain degree of fertilization competence. Further studies characterizing subpopulations could help identify key differentiation events necessary to unlock this potential or reveal molecular signatures useful for selecting and culturing the most suitable spermatids.

Flow cytometry cell sorting results indicate that we successfully isolated two distinct subpopulations of bovine spermatids based on their apparent DNA content, size and granularity. Consistent with this, epifluorescence microscopy confirmed clear morphological distinctions of the nuclei in the isolated spermatid subpopulations, typical of later stages of spermiogenesis in mammals, including human (Tesarik and Mendoza, 1996), mouse (Hasegawa et al., 2010), and cattle (Staub and Johnson, 2018). Unfortunately, as described in the result section, steps 10–12 and 13–14 spermatid stages were not efficiently separated, indicating sorting inefficiency within these gates. One possible explanation for these results may be ethanol fixation of cells prior to sorting, which could somehow affect separation and isolation of elongating and elongated spermatids. Despite this, our results are in line with a recent study demonstrating efficient flow cytometry purification of round and elongating/elongated spermatids from human and rat testis, as well as a pure population of round spermatids from mouse testis using a DNA intercalating dye (Struijk et al., 2019). Although further optimization could enhance separation efficiency, the approach described here provides useful results for generation a robust tool to enable downstream analysis of gene expression, chromatin remodeling, and other stage-specific cellular processes in spermatid development and differentiation in cattle.

5 Conclusion

Overall, our results demonstrate that the procedure described in the present manuscript significantly enhances the isolation of viable and healthy bovine spermatids, defining morphological and molecular features for their proper identification and characterization. This approach is helpful for downstream applications in embryotechnology. In addition, although spermatid injection into mature oocytes remains unsuccessful in cattle, the flow cytometry protocol set up here is valuable for

isolating highly purified spermatid subpopulations and for a better understanding of the functional and molecular aspects related to spermatid competence. Finally, this work provides useful data for developing spermatid injection protocols that might, in due course, allow to reduce the generational interval in cattle if competent spermatids are generated from embryonic cells.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

RP: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. FD: Investigation, Writing – original draft. SB: Investigation, Visualization, Writing – original draft. FFF: Investigation, Visualization, Writing – original draft. RP: Data curation, Writing – review and editing. FF: Investigation, Visualization, Writing – original draft. VL: Investigation, Visualization, Writing – original draft. AI: Data curation, Investigation, Writing – original draft, Writing – review and editing. AL: Conceptualization, Supervision, Writing – original draft. TB: Conceptualization, Supervision, Writing – original draft, Writing – review and editing. FG: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2025.1581019/full#supplementary-material

References

Abdalla, H., Shimoda, M., Hirabayashi, M., and Hochi, S. (2009). A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa. *Theriogenology* 72, 453–460. doi:10.1016/j.theriogenology.2009.03.011

Bajpai, M., Gupta, G., and Setty, B. S. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. *Eur. J. Endocrinol.* 138, 322–327. doi:10.1530/eje.0.1380322

Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M., and Dym, M. (1977). Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J. Cell. Biol.* 74, 68–85. doi:10.1083/jcb.74.1.68

Berglund, B. (2015). Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. *Infect. Ecol. Epidemiol.* 5, 28564. doi:10.3402/iee.v5.28564

Blanchard, Y., Lavault, M. T., Quernee, D., Le Lannou, D., Lobel, B., and Lescoat, D. (1991). Preparation of spermatogenic cell populations at specific stages of differentiation in the human. *Mol. Reprod. Dev.* 30, 275–282. doi:10.1002/mrd.1080300316

Cenariu, M., Pall, E., Cernea, C., and Groza, I. (2012). Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability. *J. Biomed. Biotechnol.* 2012, 1–5. doi:10.1155/2012/541384

Chen, X., Hu, T., Liang, G., Yang, M., Zong, S., Miao, S., et al. (2008). A novel testis protein, RSB-66, interacting with INCA1 (inhibitor of Cdk interacting with cyclin A1). *Biochem. Cell. Biol.* 86, 345–351. doi:10.1139/O08-072

Costa, J., Braga, P. C., Rebelo, I., Oliveira, P. F., and Alves, M. G. (2023). Mitochondria quality control and male fertility. *Biol. (Basel)* 12, 827. doi:10.3390/biology12060827

Dio, C.Di, Longobardi, V., Zullo, G., Parma, P., Pauciullo, A., Perucatti, A., et al. (2020). Analysis of meiotic segregation by triple-color fish on both total and motile sperm fractions in a t(1p;18) river buffalo bull. *PLoS One* 15, e0232592. doi:10.1371/journal.pone.0232592

Feige, E., Chen, A., and Motro, B. (2002). Nurit, a novel leucine-zipper protein, expressed uniquely in the spermatid flower-like structure. *Mech. Dev.* 117, 369–377. doi:10.1016/S0925-4773(02)00217-4

Genualdo, V., Turri, F., Pizzi, F., Castiglioni, B., Marletta, D., and Iannuzzi, A. (2021). Sperm nuclei analysis and nuclear organization of a fertile boar–pig hybrid by 2d fish on both total and motile sperm fractions. *Animals* 11, 738. doi:10.3390/ani11030738

Goszczynski, D. E., Cheng, H., Demyda-Peyrás, S., Medrano, J. F., Wu, J., and Ross, P. J. (2019). *In vitro* breeding: application of embryonic stem cells to animal production. *Biol. Reprod.* 100, 885–895. doi:10.1093/biolre/ioy256

Hall, P. F., and Mita, M. (1984). Influence of follicle-stimulating hormone on glucose transport by cultured Sertoli cells. *Biol. Reprod.* 31, 863–869. doi:10.1095/biolreprod31.5.863

Halliwell, B. (2003). Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett.* 540, 3–6. doi:10.1016/S0014-5793(03)00235-7

Hamilton, T. R. S., Simões, R., Mendes, C. M., Goissis, M. D., Nakajima, E., Martins, E. A. L., et al. (2019). Detection of protamine 2 in bovine spermatozoa and testicles. *Andrology* 7, 373–381. doi:10.1111/andr.12610

Hasegawa, H., Terada, Y., Ugajin, T., Yaegashi, N., and Sato, K. (2010). A novel culture system for mouse spermatid maturation which produces elongating spermatids capable of inducing calcium oscillation during fertilization and embryonic development. *J. Assist. Reprod. Genet.* 27, 565–570. doi:10.1007/s10815-010-9442-3

Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H., and Saitou, M. (2012). Offspring from oocytes derived from *in vitro* primordial germ cell-like cells in mice. *Science* 1979, 971–975. doi:10.1126/science.1226889

Henchion, M., Moloney, A. P., Hyland, J., Zimmermann, J., and McCarthy, S. (2021). Review: trends for meat, milk and egg consumption for the next decades and the role played by livestock systems in the global production of proteins. *Animal* 15, 100287. doi:10.1016/j.animal.2021.100287

Horiuchi, T., Emuta, C., Yamauchi, Y., Oikawa, T., Numabe, T., and Yanagimachi, R. (2002). Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: a methodological approach. *Theriogenology* 57, 1013–1024. doi:10.1016/S0093-691X(01)00701-4

Iannuzzi, A., Iannuzzi, L., and Parma, P. (2023). Molecular cytogenetics in domestic bovids: a review. *Animals* 13, 944. doi:10.3390/ani13050944

Jutte, N. H. P. M., Grootegoed, J. A., Rommerts, F. F. G., and Van Der Molen, H. J. (1981). Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. *J. Reprod. Fertil.* 62, 399–405. doi:10.1530/jrf.0.0620399

Kim, C. R., Noda, T., Okada, Y., Ikawa, M., and Baek, S. H. (2021). Protocol for isolation of spermatids from mouse testes. *Star. Protoc.* 2, 100254. doi:10.1016/j.xpro.2020.100254

Lafontaine, S., Labrecque, R., Blondin, P., Cue, R. I., and Sirard, M. A. (2023). Comparison of cattle derived from *in vitro* fertilization, multiple ovulation embryo transfer, and artificial insemination for milk production and fertility traits. *J. Dairy Sci.* 106, 4380–4396. doi:10.3168/jds.2022-22736

Li, B., Zhao, X., Jin, T., Wu, Z., and Yang, H. (2024). Efficient isolation and purification of spermatogonia, spermatocytes, and spermatids from mice, piglets, and adult boars using an optimized STA-PUT method. *Theriogenology* 213, 97–108. doi:10.1016/j.theriogenology.2023.09.023

Luciano, A. M., Franciosi, F., Lodde, V., Tessaro, I., Corbani, D., Modina, S. C., et al. (2013). Oocytes isolated from dairy cows with reduced ovarian reserve have a high frequency of aneuploidy and alterations in the localization of progesterone receptor membrane component 1 and aurora kinase b. *Biol. Reprod.* 88, 58. doi:10.1095/biolreprod.112.106856

Martins, C. F., Silva, A. E. D. F., Dode, M. N., Rumpf, R., Cumpa, H. C. B., Silva, C. G., et al. (2015). Morphological characterization and conservation of bovine spermatogenic cells by refrigeration at 4°C and freezing using different cryoprotective molecules. *Cryobiology* 71, 47–53. doi:10.1016/j.cryobiol.2015.06.003

Meistrich, M. L. (1977). Separation of spermatogenic cells and nuclei from rodent testes. *Methods Cell. Biol.* 15, 15–54. doi:10.1016/S0091-679X(08)60207-1

Modina, S., Borromeo, V., Luciano, A. M., Lodde, V., Franciosi, F., and Secchi, C. (2007). Relationship between growth hormone concentrations in bovine oocytes and follicular fluid and oocyte developmental competence. *Eur. J. Histochem.* 51, 173–180.

Murphy, M. P., Bayir, H., Belousov, V., Chang, C. J., Davies, K. J. A., Davies, M. J., et al. (2022). Guidelines for measuring reactive oxygen species and oxidative damage in cells and *in vivo*. *Nat. Metab.* 4, 651–662. doi:10.1038/s42255-022-00591-z

Ock, S. A., Kwack, D. O., Lee, S. L., Cho, S. R., Jeon, B. G., Kumar, B. M., et al. (2006a). *In vitro* development of bovine oocytes reconstructed with round spermatids. *Theriogenology* 65, 1242–1253. doi:10.1016/j.theriogenology.2005.08.005

Ock, S. A., Lee, S. L., Jeon, B. G., Cho, S. R., kumar, B. M., Choi, Y. S., et al. (2006b). Isolation and viability of presumptive spermatids collected from bull testes by Percoll density gradient. *Anim. Reprod. Sci.* 93, 144–156. doi:10.1016/j.anireprosci.2005.07.011

Pasquariello, R., Bogliolo, L., Di Filippo, F., Leoni, G. G., Nieddu, S., Podda, A., et al. (2024). Use of assisted reproductive technologies (ARTs) to shorten the generational interval in ruminants: current status and perspectives. *Theriogenology* 225, 16–32. doi:10.1016/J.THERIOGENOLOGY.2024.05.026

Pasquariello, R., Ermisch, A. F., Silva, E., McCormick, S., Logsdon, D., Barfield, J. P., et al. (2018). Alterations in oocyte mitochondrial number and function are related to spindle defects and occur with maternal aging in mice and humans. *Biol. Reprod.* 100, 971–981. doi:10.1093/biolre/ioy248

Pelengaris, S. A., and Moore, H. D. M. (1995). Separation of round spermatids from the rat using an immunoselection panning technique. *Mol. Reprod. Dev.* 41, 348–354. doi:10.1002/mrd.1080410310

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29, 45e–45e. doi:10.1093/nar/29.9.e45

Reuben, J. M., and Bó, G. A. (2020). *Chapter 1a. General sanitary procedures and considerations associated with in vivo-derived bovine embryos*. Fifth.

Ritchie, H., and Roser, M. (2024). Yields vs. land use: how the Green Revolution enabled us to feed a growing population. *Our World Data*. Available online at: https://ourworldindata.org/yields-vs-land-use-how-has-the-world-produced-enough-food-for-a-growing-population.

Simard, O., Leduc, F., Acteau, G., Arguin, M., Grégoire, M. C., Brazeau, M. A., et al. (2015). Step-specific sorting of mouse spermatids by flow cytometry. *J. Vis. Exp.* 2015, e53379. doi:10.3791/53379

Staub, C., and Johnson, L. (2018). Review: spermatogenesis in the bull. *Animal* 12, s27–s35. doi:10.1017/S1751731118000435

Steger, K. (1999). Transcriptional and translational regulation of gene expression in haploid spermatids. *Anat. Embryol. Berl.* 199, 471–487. doi:10.1007/s004290050245

Struijk, R. B., De Winter-Korver, C. M., van Daalen, S. K. M., Hooibrink, B., Repping, S., and van Pelt, A. M. M. (2019). Simultaneous purification of round and elongated spermatids from testis tissue using a FACS-based DNA ploidy assay. *Cytom. Part A* 95, 309–313. doi:10.1002/cyto.a.23698

Tanaka, A., Nagayoshi, M., Takemoto, Y., Tanaka, I., Kusunoki, H., Watanabe, S., et al. (2015). Fourteen babies born after round spermatid injection into human oocytes. *Proc. Natl. Acad. Sci. U. S. A.* 112, 14629–14634. doi:10.1073/pnas.1517466112

Tesarik, J., and Mendoza, C. (1996). Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. *Hum. Reprod.* 11, 772–779. doi:10.1093/oxfordjournals.humrep.a019253

Willison, K. R., Hynes, G., Davies, P., Goldsborough, A., and Lewis, V. A. (1990). Expression of three t-complex genes, Tcp-1, D17Leh117c3, and D17Leh66, in purified murine spermatogenic cell populations. *Genet. Res.* 56, 193–201. doi:10.1017/S0016672300035291

Xu, J., Lu, H., Li, H., Yan, C., Wang, X., Zang, M., et al. (2021). Computerized spermatogenesis staging (CSS) of mouse testis sections via quantitative histomorphological analysis. *Med. Image Anal.* 70, 101835. doi:10.1016/j.media.2020.101835

Zhu, H., Jiang, Y., Quan, H., Li, L., Wei, J., Liu, R., et al. (2024). Mouse round spermatid injection. *J. Vis. Exp.* 2024. doi:10.3791/65898

*CHAPTER 4: **Creation of testicular bio-scaffolds***

Aim of the chapter

The complete *in vitro* replication of spermatogenesis remains an unresolved challenge, particularly in livestock species. Spermatogenesis is a highly intricate and tightly regulated biological process, and its successful reproduction under laboratory conditions has proven especially difficult in domestic animals. To overcome these limitations and advance the field, the development of reliable three-dimensional (3D) *in vitro* models that accurately recapitulate the native architecture and physiological microenvironment of the testis is essential. These models offer a promising strategy to support and enhance the generation of haploid male gametes under controlled conditions.

3D culture systems represent a more physiologically relevant alternative to traditional two-dimensional (2D) platforms. Unlike 2D systems, which fail to replicate the complex spatial organization and dynamic microenvironment of native tissues, 3D models provide structural support, cell–to-cell and cell–to-matrix interactions, and tissue-specific biochemical cues that closely mimic *in vivo* conditions (Brevini et al., 2020; Pennarossa et al., 2021; Xu et al., 2022).

Several 3D strategies have already been applied in reproductive biology, including synthetic culture supports such as permeable membrane insert (Brocke et al., 2024; Knight & Przyborski, 2015)and 3D porous polystyrene scaffold (Costello et al., 2021; Díez et al., 2023; J. Li et al., 2019; Pennarossa et al., 2024; Staun-Ram & Shalev, 2005; Velicky et al., 2016), decellularized biological scaffolds (Di Filippo et al., 2024; Pennarossa et al., 2020; Pennarossa et al., 2020), hanging drop cultures (Rasouli et al., 2024) and microbioreactors (Aussillous & Quéré, 2001; Nguyen et al., 2020; Pennarossa et al., 2020). These platforms enable detailed studies on implantation, gametogenesis, and reproductive disorders, while also supporting applications in fertility preservation and regenerative medicine (Pennarossa et al., 2023; Pennarossa et al., 2021; Pennarossa et al., 2020; Pennarossa et al., 2020).

Based on the above, we believe that 3D platforms hold significant potential for facilitating *in vitro* gametogenesis, thereby contributing to the reduction of generation intervals in livestock. Accordingly, this chapter describes the development of bovine testicular bioscaffolds and demonstrates their successful *in vitro* repopulation with bovine testicular fibroblasts.

Specifically, the experiments presented in the article describe the development of a testis-specific decellularization protocol, that combines

physical and chemical methods to remove the cellular compartment, while preserving the architecture and composition of the extracellular matrix (ECM). The efficiency of cell removal and the preservation of key ECM components, namely collagen, elastin, and glycosaminoglycans, were assessed using specific staining techniques and stereological analyses. The obtained bio-scaffolds were then repopulated with previously isolated bovine testicular fibroblasts. The results demonstrated that the decellularized scaffolds supported cell adhesion and proliferation, confirming that ECM functionality was retained.

These findings suggest that the generated 3D bio-scaffolds may provide an optimal artificial niche for testicular cell culture and represent a promising platform for the *in vitro* spermatogenesis in domestic species.

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Generation of bovine decellularized testicular bio-scaffolds as a 3D platform for testis bioengineering

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Accelerating the genetic selection to obtain animals more resilient to climate changes, and with a lower environmental impact, would greatly benefit by a substantial shortening of the generation interval. One way to achieve this goal is to generate male gametes directly from embryos. However, spermatogenesis is a complex biological process that, at present, can be partially reproduced *in vitro* only in the mouse. The development of reliable 3D *in vitro* models able to mimic the architecture and the physiological microenvironment of the testis, represents a possible strategy to facilitate *ex vivo* haploid male gamete generation in domestic species. Here we describe the creation of bovine testicular bio-scaffolds and their successful repopulation *in vitro* with bovine testicular cells. In particular, bovine testes are subjected to three different decellularization protocols. Cellular compartment removal and extracellular matrix preservation are evaluated. The generated bio-scaffolds are then repopulated with bovine testicular fibroblasts. The results obtained demonstrate that the decellularization protocol involving the use of 0.3% sodium dodecyl sulfate (SDS) for 12 h efficiently eliminates native cells, while preserving intact ECM composition and microstructure. Its subsequent repopulation with bovine fibroblasts demonstrates successful cell homing, colonization and growth, consistent with the scaffold ability to sustain cell adherence and proliferation. Overall, the generated 3D bio-scaffolds may constitute a suitable artificial niche for *ex vivo* culture of testicular cells and may represent a possible strategy to reproduce spermatogenesis *in vitro*.

KEYWORDS

3D bio-scaffold, bovine, decellularization, extracellular matrix, testis

1 Introduction

The obtainment of animals more resilient to climate changes, and with a lower environmental impact, would be greatly desirable. Based on this, during the last years, particular attention has been dedicated to the development and further improvement of a sustainable animal agriculture with positive socioeconomic and environmental impacts. In this perspective, new biotechnological approaches, such as assisted reproductive technologies (ART) and genetic selection (GS), were introduced and used simultaneously in livestock breeding programs, including in the bovine species (Mueller

and Van Eenennaam, 2022). Nevertheless, the possibility of these techniques to improve genetic gain is limited by the average age of an animal when replacement offspring are born (Kasinathan et al., 2015). It is therefore clear that the greatest improvement in accelerating the genetic selection can be achieved by shortening the generation interval (Cenariu et al., 2012; Pasquariello et al., 2024). To this aim, different strategies have been proposed in both male and female domestic ruminants, however, a substantial shortening of the interval is still far away.

In the male, one promising approach is represented by the *in vitro* propagation and differentiation of spermatogonia into mature sperm or even *in vitro* recreation of whole spermatogenesis from embryonic stem cells. To date, a variety of culture systems as well as different medium compositions to enhance spermatogonial stem cell (SSC) viability, proliferation (Fath-Bayati et al., 2023; van Maaren et al., 2023) and differentiation *in vitro* (Cho and Easley, 2023; Kulibin and Malolina, 2023; Salem et al., 2023; Damyanova et al., 2024) have been developed. However, the complete spermatogenesis *ex vivo* has been obtained only in the murine species (Perrard et al., 2016), while, in cattle and in other domestic species, it is possible only to propagate spermatogonia without inducing an effective meiotic division. This is mainly due to the complexity of the process, during which male germ cells differentiate into mature spermatozoa, thank to well-orchestrated interactions among hormones, growth factors, cytokines, and extracellular matrix (ECM)-derived bio-mechanical and bio-chemical cues. In addition, the lack of knowledge on niche microenvironment, nutritional requirements, as well as on the multiple regulatory machinery driving self-renewal, proliferation, and differentiation, in ruminates, has significantly hindered progresses in this field. It is therefore desirable to developed reliable 3D *in vitro* models able to faithfully mimic the architecture and the physiological microenvironment of the native testicular tissue, bridging the gap between the *in vivo* complexity and the over-simplified conventional two-dimension (2D) *in vitro* cultures.

To date, several 3D platforms for testicular bioengineering, including testicular organoids (Richer et al., 2020), hydrogel bioreactors (Perrard et al., 2016), and synthetic, natural or decellularized scaffolds (Horvath-Pereira et al., 2023) have been developed in human, mouse and rat. In contrast, to our knowledge, no cell engrafted 3D scaffolds have been developed in the bovine species and only one study reported the differentiation of gonocytes into presumptive spermatids through the use the alginate encapsulation technique (Lee et al., 2001). In this scenario, the generation of a bovine decellularized testicular 3D scaffold represents a promising option, since it accurately replicates the *in vivo* topography and the complex milieu of the native tissue, thus promoting the necessary interactions between cells and their surrounding microenvironment. In addition, the preserved extracellular matrix (ECM), obtained through the decellularization process, provides essential biomechanical and biochemical cues that encourage the correct cell growth, differentiation, and function.

In the present study, we generate bovine testicular bio-scaffolds for the creation of reliable 3D artificial models. In particular, we test three different decellularization protocols and identify the protocol that better preserves intact ECM composition and microstructure, while efficiently eliminating cells. We then repopulate the generated testicular bio-scaffolds with fibroblasts isolated from bovine testes and monitor the scaffold ability to sustain cell adherence and proliferation.

2 Materials and methods

All reagents were purchased from Thermo Fisher Scientific unless otherwise indicated.

2.1 Ethic statement

Bovine testes were collected at the local abattoir from adult animals. Organs were isolated from animals destined to human consumption and, therefore, were not considered as animal experimentation under Directive 2010/63/EU of the European Parliament. All experiments were performed in accordance with the approved guidelines.

2.2 Testis collection

Organs were collected from four 2 years-old bulls at the local slaughterhouse and transported to the laboratory in cold sterile saline solution (NaCl 0.9%) within 1 hour. Testes were washed in phosphate-buffered saline (PBS), decapsulated and cut in small pieces of 0.5 × 0.5 × 0.5 cm^3. Fragments were randomly allocated to four experimental groups: untreated tissue, control group, (CTR; n = 4), decellularization protocol A (Decell-A; n = 52); decellularization protocol B (Decell-B; n = 52), decellularization protocol C (Decell-C; n = 52). Untreated tissue samples, used ad control group (CTR), were immediately fixed in 10% buffered formalin (Bio-Optica) for histological evaluations. The other groups were subjected to the three different decellularization processes as described below.

2.3 Decellularization protocols

Testicular fragments belonging the experimental groups Decell-A, Decell-B, and Decell-C were frozen at −80°C for at least 24 h, thawed at 37°C in a water bath for 30 min, and decellularized in:

A. 0.3% (v/v) sodium dodecyl sulfate (SDS; Bio-Rad) in deionized water (DI-H2O) for 6 h and then in 1% (v/v) Triton X-100 in DI-H2O for 6 h;
B. 0.3% (v/v) sodium dodecyl sulfate (SDS; Bio-Rad) in DI-H2O for 12 h and then in 1% (v/v) Triton X-100 in DI-H2O for 6 h;
C. 0.3% (v/v) sodium dodecyl sulfate (SDS; Bio-Rad) DI-H2O for 24 h and then in 1% (v/v) Triton X-100 in DI-H2O for 6 h.

At the end of the decellularization protocols, testes were washed in DI-H2O for 6 h with changes every 2 h. All steps were carried out using an orbital shaker at 150 rpm at room temperature. At the end of the procedures, from each experimental group, samples were fixed

for histology and stained with hematoxylin and eosin (H&E, Bio-Optica), Crossmon Trichrome (Bio-optica), Alcian blue (pH 1; Bio-optica) Orcein and 4,6-diamidino-2-phenylindole (DAPI), or used for *in vitro* re-seeding studies. Cell density analysis and stereological evaluations were then performed at least in triplicates.

2.4 Histological evaluations

Samples were fixed in 10% buffered formalin for 24 h at room temperature, dehydrated in graded alcohols, cleared with xylene, embedded in paraffin, and cut in serial microtome sections (5 μm thick). The latters were dewaxed, re-hydrated and stained with H&E (Bio-Optica), Crossmon Trichrome (Bio-optica), Alcian blue (pH 1; Bio-optica) and Orcein, in agreement with previously published studies (Verdile et al., 2022; Khazaei et al., 2023). Samples were analyzed under an Eclipse E600 microscope (Nikon) equipped with a digital camera (Nikon). Pictures were acquired with NIS-Elements Software (Version 4.6; Nikon). Untreated testicles were used as the control.

2.5 Cell density

Cell density analyses were carried out as previously described (Pennarossa et al., 2020). More in detail, serial microtome sections (5 μm thick) were cut, dewaxed, re-hydrated and stained with DAPI. Cell number was quantified in 5 tissue sections obtained from each testis (n = 4) subjected to the three different decellularization protocol (A, B, and C) and from 4 CTR testis. Within each section, 5 randomly selected fields at ×100 total magnifications were analyzed. Samples were analyzed under an Eclipse E600 microscope (Nikon) equipped with a digital camera (Nikon). Images were captured with NIS-Elements Software (Version 4.6; Nikon) and analyzed, using the Cell Counter plugin of the image analysis software ImageJ, following the instructions. Briefly, 8-bit grayscale images were generated applying threshold adjustments and segmented using a thresholding algorithm to highlight the areas occupied by the nuclei and remove the background. The data obtained were transformed into binary format. Size and circularity parameters were defined, and the nuclei were automatically enumerated. Untreated testicles were used as the control.

2.6 Stereological analyses

Collagen, elastin, and GAG volume density (Vv) evaluations were performed on sections stained with Crossmon Trichrome, Orcein and Alcian blue, respectively. As described by Albl et al. (2016), the Delesse principle was used, and the proportional volume of each specific area was calculated as the fraction of the structure of interest (e.g., collagen) relative to the total area of the reference compartment (e.g., whole section). Images were randomly taken, overlaid with a point-count stereological grid containing evenly spaced test points and the relative volume of each region of interest was calculated by dividing the number of points striking the structure of interest by the number of points hitting the reference compartment. Vv was expressed as percentages using the following formula:

$$Vv\ (_{\text{analyzed compartment,reference compartment}}) = \left[\sum P(\text{analyzed compartment}) \Big/ \sum P(\text{reference compartment})\right] \times 100$$

$\sum P\ (\text{analyzed compartment})$: the number of points hitting the compartment under study;
$\sum P\ (\text{reference compartment})$: the number of points hitting the relevant structure.

2.7 Bovine testicular fibroblast isolation

Adult bovine fibroblasts were isolated from fresh testicular tissues obtained from 3 individuals (Albrecht et al., 2006). Testes were decapsulated and cut into small fragments of ~ 2 mm^3. These were placed into 35 mm^2 Petri dishes (Sarstedt) previously coated with 0.1% gelatin (Sigma-Aldrich). Droplets of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS, 2 mM glutamine (Sigma-Aldrich), and 2% antibiotic/antimycotic solution (Sigma-Aldrich) were added onto each fragment. Culture dishes were incubated in 5% CO2 at 37°C in humidified chambers. After 4 days of culture, bovine testicular fibroblasts started to grow out of the original explants, and the latter were carefully removed. Fibroblasts were cultured using the medium described above, grown in 5% CO2 at 37°C, and passaged twice a week at a 1:3 ratio. The three bovine primary cell lines were used in triplicate in 3 independent experiments.

2.8 Bio-scaffold repopulation with bovine testicular fibroblasts

Testicular bio-scaffolds obtained from Decell-A, Decell-B, and Decell-C groups were sterilized with 70% ethanol and 2% Penicillin/Streptomycin/Amphotericin B solution in sterile H_2O for 30 min, extensively washed in sterile PBS and equilibrated in DMEM for 1 h at 37°C. Scaffolds of 0.5 × 0.5 cm^2 and 1 mm thick were obtained using sharp scalpel and placed into a 4-well multidishes (1 fragment per well; Nunc). 0.5 × 10^6 of bovine testicular fibroblasts were resuspended in 100 μL of DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine (Sigma-Aldrich), and 1% antibiotic/antimycotic solution (Sigma-Aldrich), seeded onto each scaffold and co-cultured at 37°C incubator with 5% CO2. Re-seeding density was selected based on our previous studies (Pennarossa et al., 2020; Pennarossa et al., 2021b; Arcuri et al., 2024). Half medium volume was changed every 2 days. Cultures were arrested for histological evaluations at days 1, 3 and 7. All experiments were performed in triplicates.

2.9 Statistical analysis

Statistical analysis was performed using ANOVA with Tukey's *post hoc* (SPSS 19.1; IBM). At least three experiments were carried out for all analyses. Data were reported as mean ± standard error of

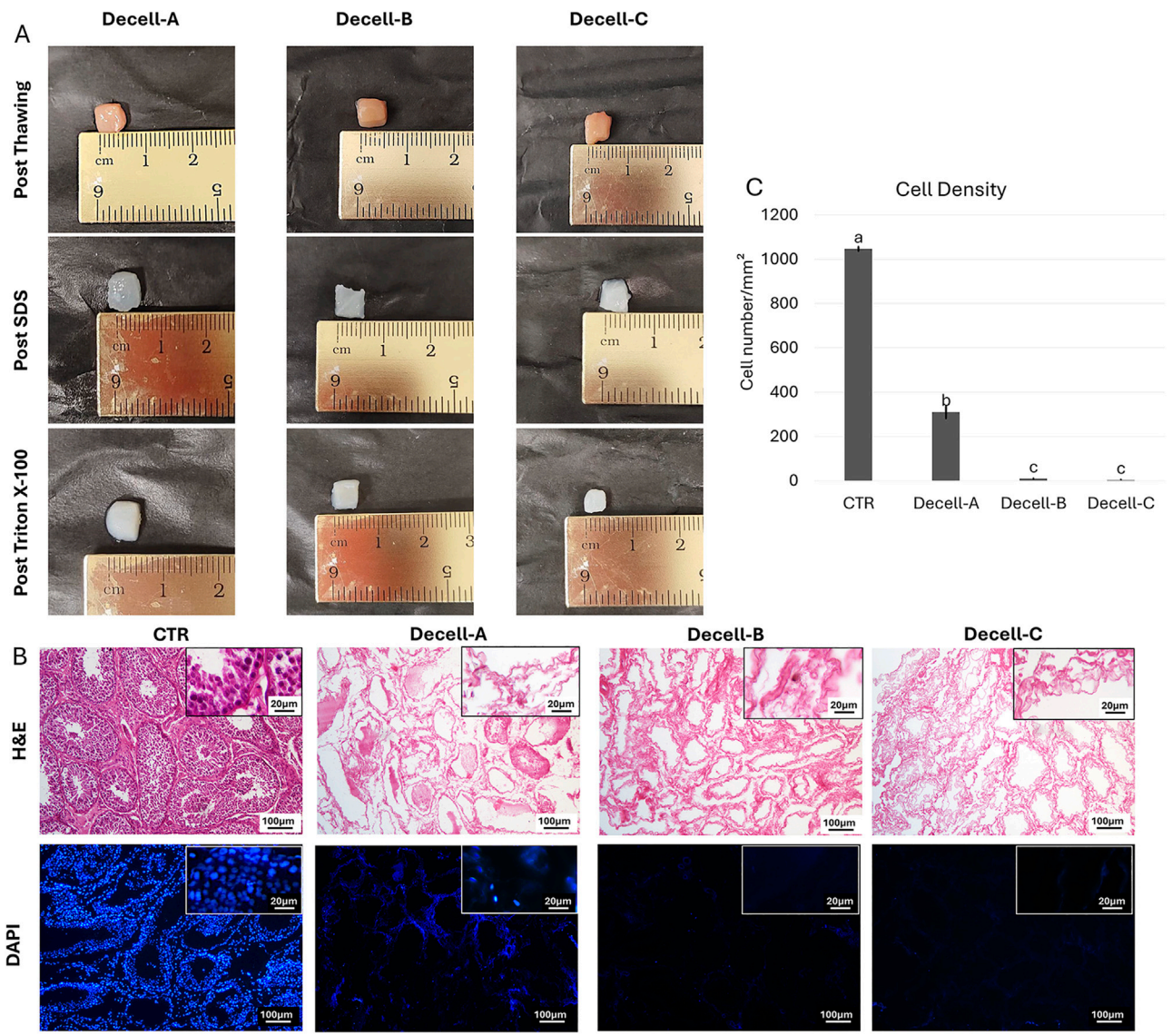

FIGURE 1
Macro/microscopic evaluations of decellularized testes. (A) Three-step macroscopic images illustrating the color of samples that turns from red to white, regardless of the decellularization protocol used. **(B)** H&E staining shows the presence of both basophilic (cell nuclei) and eosinophilic (cell cytoplasm and ECM) staining in untreated tissue (CTR) and their absence in decellularized testes. DAPI staining displaying the presence of nuclei in CTR samples and their disappearance after the decellularization processes. **(C)** Cell density analysis demonstrates a significantly lower number of nuclei in all decellularized tissues compared with the untreated one (CTR). Data are expressed as the mean ± standard error of the mean (SEM). A, b, c Different superscripts indicate $p < 0.05$.

the mean (SEM). Differences of $p \leq 0.05$ were considered significant.

3 Results

3.1 Testicular bio-scaffold evaluation

3.1.1 Macroscopic assessments

Macroscopic observations revealed that, during the decellularization process, the color of the testicular fragments gradually turned from red to white, regardless to the decellularization protocol used (Decell-A, Decell-B, and Decell-C, Figure 1A).

3.1.2 Histological analysis of cell nuclei and tissue after the different decellularization protocols

Histological assessments demonstrated that all the three decellularization protocols tested removed cells (Figure 1B). In particular, H&E staining showed the decrement of basophilic staining in Decell-A, Decell-B, and Decell-C (Figure 1B), while both the basophilic and eosinophilic staining were visible in the untreated tissue (CTR, Figure 1B). DAPI staining and cell density analysis confirmed these observations, indicating a significantly

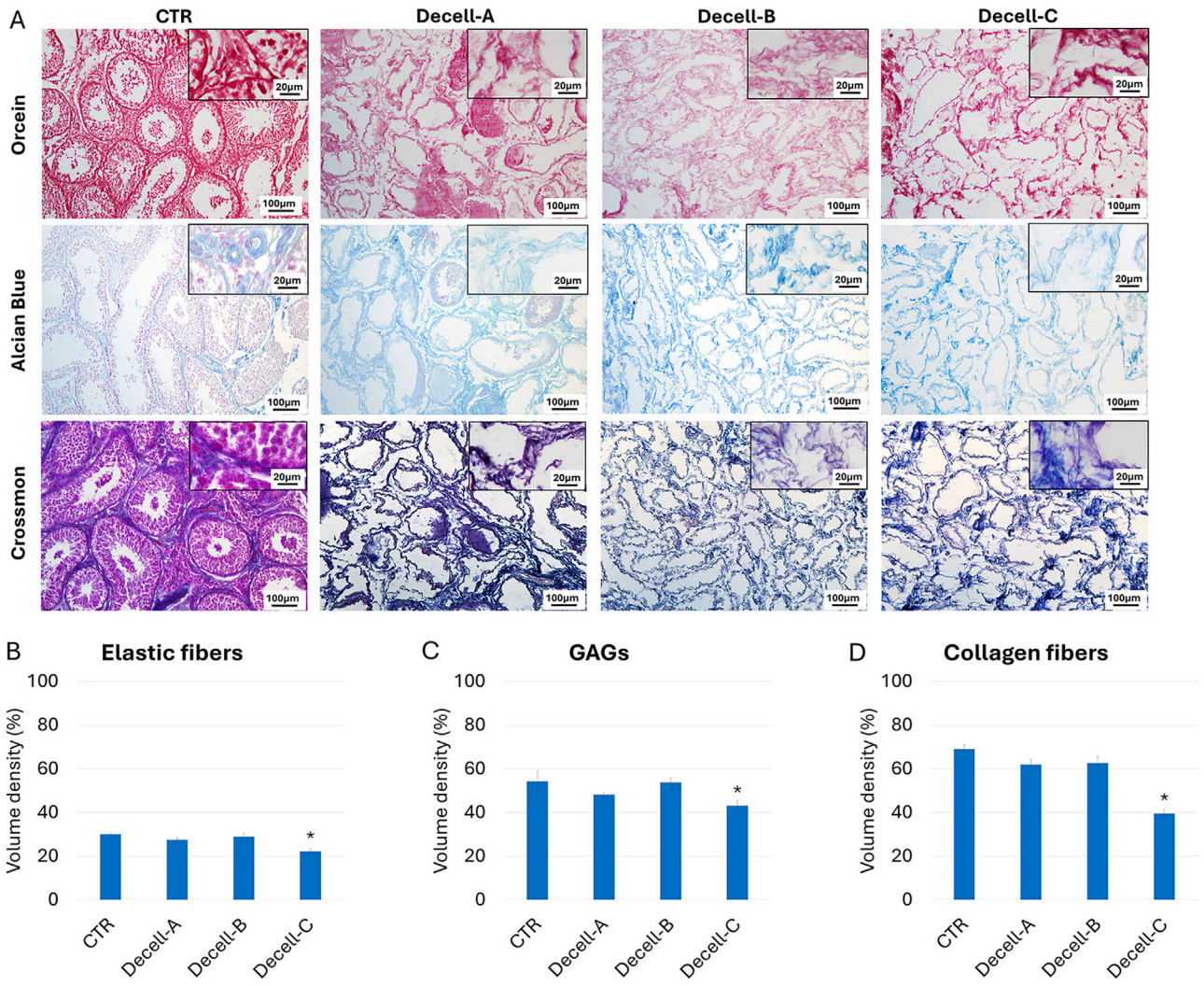

FIGURE 2
ECM microarchitecture and composition in untreated (CTR) and decellularized testes. **(A)** Orcein, alcian blue and Crossmon's trichrome staining indicate the preservation of elastic fibers, GAGs, and collagen, respectively, in Decell-A and Decell-B, while a reduction for all the ECM components is visible in Decell-C. **(B)** Elastic fiber stereological quantification shows comparable amount of the protein in untreated tissue (CTR), Decell-A and Decell-B, while a significant decrement is detected in Decell-C. Data are expressed as the mean ± standard error of the mean (SEM) (*$p > 0.05$). **(C)** GAG stereological analysis displays a significant reduction in Decell-C compared to Decell-A, Decell-B and CTR. Data are expressed as the mean ± standard error of the mean (SEM) (*$p > 0.05$). **(D)** Collagen content quantification demonstrates comparable amount of the fibers in untreated tissue (CTR), Decell-A and Decell-B, while a significant decrement is detected in Decell-C. Data are expressed as the mean ± standard error of the mean (SEM) (*$p > 0.05$).

lower number of nuclei in all the obtained bio-scaffolds, when compared with the untreated tissues (Figures 1B, C). However, cell density analysis demonstrated that Decell-B and Decell-C protocol removed the cellular compartment more efficiently than Decell-A (Figure 1C).

Orcein staining (Figure 2A) showed the preservation of intact elastic fibers after Decell-A and Decell-B, with a comparable distribution of elastin among Decell-A, Decell-B, and CTR samples. In contrast, a reduction was detected in Decell-C at the end of the process. These morphological observations were consistent with elastin stereological quantifications, which indicated a significant elastin decrement in the Decell-C group (Figure 2B). Similarly, Alcian Blue staining showed glycosaminoglycan (GAG) retention in Decell-A and Decell-B, which exhibited a GAG distribution comparable to that of the untreated tissue (CTR, Figure 2A). In contrast, a decrease was observed in Decell-C. Consistent with this, stereological analysis displayed statistically significant changes in total GAG content in Decell-C compared to the tissue of origin (Figure 2C). Crossmon trichrome staining demonstrated the persistence of collagen fibers in Decell-A and Decell-B samples, while a reduction was detected in Decell-C (Figure 2A). In agreement with these observations, stereological studies showed a statistically significant decrement of collagen fibers in Decell-C group when compared to CTR (Figure 2D).

3.2 Repopulation of the generated bio-scaffolds with bovine testicular fibroblasts

Bovine testicular fibroblasts rapidly adhered and colonized Decell-A, Decell-B, and Decell-C within 24 h of co-culture

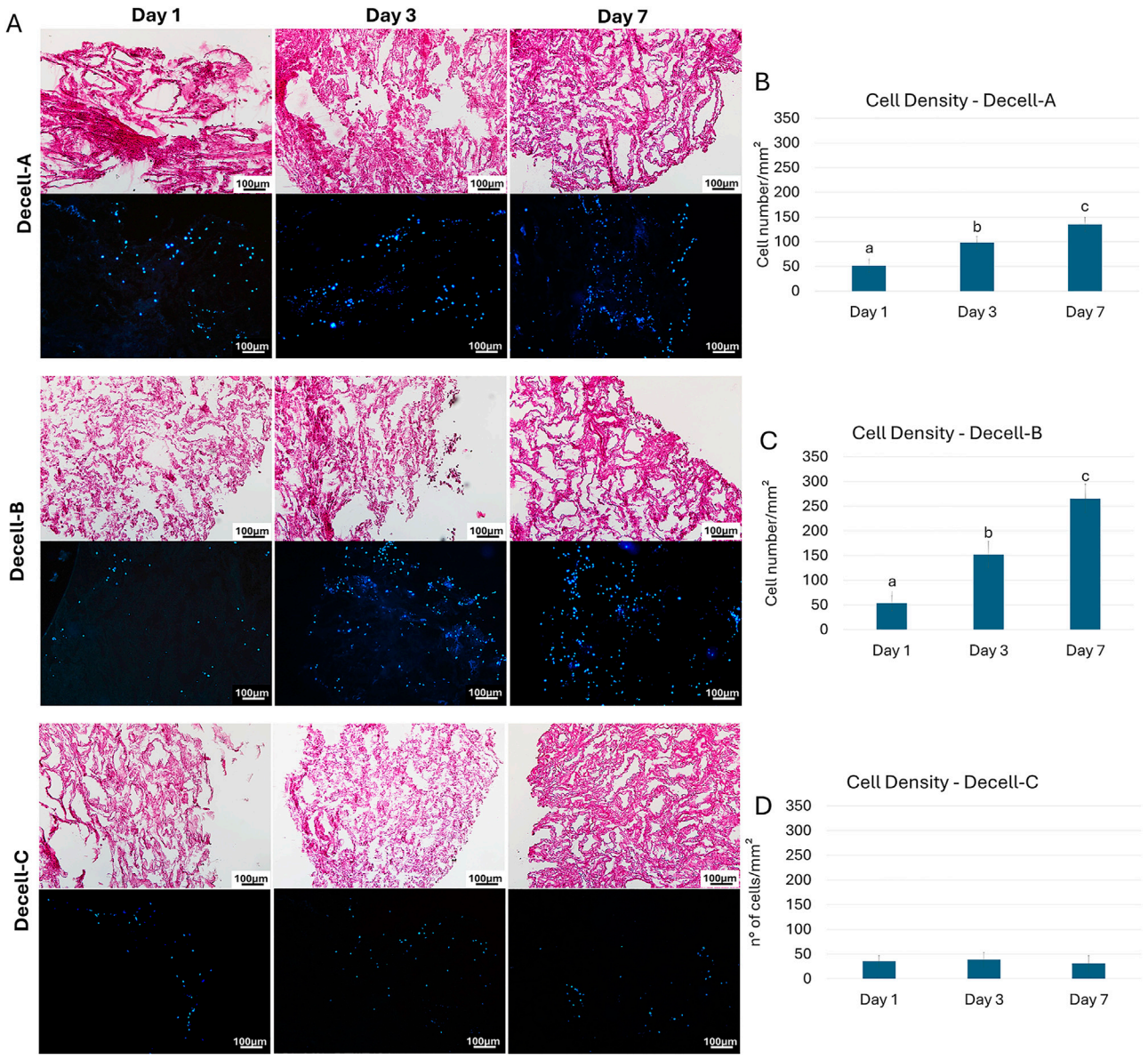

FIGURE 3
Re-seeding of decellularized testicular tissue. **(A)** H&E and DAPI staining demonstrate the presence of cells into the bio-scaffolds after 24 h from seeding (Day 1) in all the experimental groups. An increment during the following days (Day 3 and Day 7) is visible in Decell-A and Decell-B. **(B–D)** Cell density analyses confirm bio-scaffold re-population after 24 h (Day 1), with a significantly higher cell number at Day 3 and Day 7 in Decell-A and Decell-B. Decell-C shows comparable values at each time point considered. Data are expressed as the mean ± standard error of the mean (SEM). A, b, c, Different superscripts denote significant differences ($p < 0.05$).

(Figure 3A). H&E and DAPI staining showed an increasing number of cells during the following days for Decell-A and Decell-B, while no increment was visible in the Decell-C group (Figure 3A). These observations were further supported by cell density analysis that indicated the presence of cells into the bio-scaffolds 24 h after seeding (Day 1, Figures 3B–D) and a statistically significant cell number increment in Decell-A and Decell-B during the subsequent days of culture (Day 3 and Day 7, Figures 3B, C). No cell number increase was detected in the Decell-C during the 7-day culture period (Figure 3D).

4 Discussions

In the present manuscript, we generate a testicular bio-scaffold, able replicate the *in vivo* topography and the bio-mechanical and bio-chemical stimuli derived from the native ECM. The decellularized bio-scaffold successfully encourages cell homing, colonization and growth, demonstrating its ability to sustain testicular cell adherence and proliferation.

At the end of the three-step decellularization process, macroscopic evaluations revealed a color change from red to white in all the obtained bio-scaffolds, regardless of the protocol used. This suggests

the occurrence of a significant reduction in the cellular components. Indeed, similar color variations were previously reported by other Authors, which applied decellularization protocols to different tissue, including heart (Rajabi-Zeleti et al., 2014), lung (Lecht et al., 2014), liver (Ghiringhelli et al., 2021; Lee et al., 2017), kidney (Yu et al., 2014), muscle (Aulino et al., 2015), trachea (Baiguera et al., 2014; Pennarossa et al., 2021b), esophagus (Sjöqvist et al., 2014), urinary tissue (Singh et al., 2018), arteries (Kajbafzadeh et al., 2017), derma (Gilpin and Yang, 2017), intestine (Arcuri et al., 2024), ovary (Laronda et al., 2015; Laronda, 2020; Pennarossa et al., 2020; Pennarossa et al., 2022) and vagina (Zhang et al., 2017), and that resulted in a significant decrease in cell content. This was confirmed by our H&E staining that demonstrated a decrease of basophilic color in all the three experimental groups and by DAPI staining, showing a significant decrement in cell nuclei in Decell-A, Decell-B, and Decell-C samples. All these morphological observations were further corroborated by cell density analysis experiments that indicated a statistically significant cell number reduction in all experimental groups, when compared with the untreated tissues (CTR). However, while the cellular compartment was reduced in all three groups, it is interesting to note that Decell-B and Decell-C protocols allowed a more efficient and significant reduction in cell number, compared to Decell-A. Altogether, these results demonstrate that the correct combination of a freeze-thaw cycle, with sequential incubations with SDS and Triton X-100, allows for an efficient cell removal only when the native tissue is exposed to SDS for at least 12 h, as in the protocols Decell-B and Decell-C. This is in agreement with previous studies which demonstrated SDS ability to successfully eliminate the cellular compartment from the native tissues (Scarrit et al., 2015; Singh et al., 2023), selecting in a tissue-specific manner the appropriate concentration and time of exposure (Gilpin and Yang, 2017).

It is however important to note that a fundamental aspect in the decellularization process is also the maintenance of the original ECM microstructures, including fibers and macromolecules. The histochemical analysis carried out in our study, demonstrated the preservation of intact elastic fibers, GAGs and collagen in Decell-A and Decell-B. This is in agreement with Kiani et al. (2021) and Khazaei et al. (2023) that described the generation of rat and calf testis scaffolds, respectively, and demonstrated the persistence of the major ECM proteins at the end of the decellularization process. In contrast, elastic and collagen fibers, as well as GAGs appeared to be significantly reduced when SDS exposure is prolonged (Decell-C). All these morphological observations were also confirmed by stereological analysis, which revealed no significant changes for collagen, elastin, and GAG content among the untreated tissue, Decell-A and Decell-B groups. In contrast, Decell-C showed a statistically significant decrement in the ECM components when compared to the CTR. Although several different explanations can be hypothesized, we suggest that the prolonged exposure to SDS used in the Decell-C protocol, while ensuring an efficient removal of the cellular compartment, may exert a detrimental effect and cause damages to structural proteins, such as collagen fibers and GAGs. This is in line with previous observations that described SDS disruptive side effects on collagen fibers in porcine urinary bladder (Faulk et al., 2014), caprine pancreas (Singh et al., 2023) and many other tissues (Crapo et al., 2011; Keane et al., 2015). It is also in agreement with Kasturi and Vasanthan and Moffat et al. that reported SDS ability to damage GAGs during the decellularization processes of different organs, including liver, pericardium, articular cartilage, heart, and kidney (Moffat et al., 2022; Kasturi and Vasanthan, 2023). All these observations clearly point to the need of a strategy that sets a fine tuning of the SDS conditions to ensure, in a species-specific and organ-specific way, the efficient removal of cells, while preserving an intact ECM structure.

A crucial point for the use of a bio-scaffold in tissue engineering is its ability to encourage cell adhesion, homing, and growth (Chan and Leong, 2008; Lynch et al., 2021; Pennarossa et al., 2021a). To address this point, we isolated fibroblasts from bovine testis and used them to repopulate the generated decellularized bio-scaffolds. The results obtained demonstrated a rapid engrafting process with cells that adhered and colonized the matrix within 24 h from seeding. In addition, H&E, DAPI staining and cell density analysis showed a linear increment in cell number and a homogenous and steady distribution of the cell population onto Decell-A and Decell-B scaffolds for as long as 7 days, when culture was arrested. It is also interesting to note that, although the number of cells identified in Decell-A should be affected by cellular residues present at the end of the decellularization process, the increasing number observed during the culture period demonstrates the presence of proliferating cells which derive from reseeding process. Altogether, these results indicate the bio-scaffold ability to host cells and to encourage their proliferation possibly via bio-mechanical and bio-chemical stimuli. They also exclude the persistence of toxic carry-overs from the decellularization processes, may impair the subsequent recellularization and biocompatibility, both *in vitro* and *in vivo* (Morris et al., 2017).

Overall, the results obtained suggest the potential of the bio-scaffold here described to sustain cell adherence and proliferation. In particular, the cell type selected for repopulation experiments well fits with the possible use of iPS-derived or chemically reprogrammed cells, for regenerative experiments of the testicular tissues. Although, these results are still preliminary, they pave the way toward the use of decellularized testicular bio-scaffolds in the field of reproductive biology and biotechnology as suitable artificial niches for *ex vivo* culture of testicular cells and to reproduce spermatogenesis *in vitro*.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because Organs were collected from animals destined to human consumption and, therefore, were not considered as animal experimentation under Directive 2010/63/EU of the European Parliament.

Author contributions

FD: Data curation, Investigation, Methodology, Writing–review and editing. TB: Conceptualization, Data curation, Writing–review

and editing. GP: Conceptualization, Funding acquisition, Supervision, Writing–original draft. FG: Funding acquisition, Project administration, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Albl, B., Haesner, S., Braun-Reichhart, C., Streckel, E., Renner, S., Seeliger, F., et al. (2016). Tissue sampling guides for porcine biomedical models. *Toxicol. Pathol.* 44, 414–420. doi:10.1177/0192623316631023

Albrecht, M., Rämsch, R., Köhn, F. M., Schwarzer, J. U., and Mayerhofer, A. (2006). Isolation and cultivation of human testicular peritubular cells: a new model for the investigation of fibrotic processes in the human testis and male infertility. *J. Clin. Endocrinol. Metab.* 91, 1956–1960. doi:10.1210/JC.2005-2169

Arcuri, S., Pennarossa, G., Prasadani, M., Gandolfi, F., and Brevini, T. A. L. (2024). Use of decellularized bio-scaffolds for the generation of a porcine artificial intestine. *Methods Protoc.* 7, 76. doi:10.3390/mps7050076

Aulino, P., Costa, A., Chiaravalloti, E., Perniconi, B., Adamo, S., Coletti, D., et al. (2015). Muscle extracellular matrix scaffold is a multipotent environment. *Int. J. Med. Sci.* 12, 336–340. doi:10.7150/ijms.10761

Baiguera, S., Del Gaudio, C., Kuevda, E., Gonfiotti, A., Bianco, A., and Macchiarini, P. (2014). Dynamic decellularization and cross-linking of rat tracheal matrix. *Biomaterials* 35, 6344–6350. doi:10.1016/J.BIOMATERIALS.2014.04.070

Cenariu, M., Pall, E., Cernea, C., and Groza, I. (2012). Evaluation of bovine embryo biopsy techniques according to their ability to preserve embryo viability. *J. Biomed. Biotechnol.* 2012, 1–5. doi:10.1155/2012/541384

Chan, B. P., and Leong, K. W. (2008). Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *Eur. Spine J.* 17, 467–479. doi:10.1007/s00586-008-0745-3

Cho, I. K., and Easley, C. A. (2023). Recent developments in *in vitro* spermatogenesis and future directions. *Reprod. Med.* 4, 215–232. doi:10.3390/REPRODMED4030020

Crapo, P. M., Gilbert, T. W., and Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials* 32, 3233–3243. doi:10.1016/j.biomaterials.2011.01.057

Damyanova, K. B., Nixon, B., Johnston, S. D., Gambini, A., Benitez, P. P., and Lord, T. (2024). Spermatogonial stem cell technologies: applications from human medicine to wildlife conservation. *Biol. Reprod.* 111, 757–779. doi:10.1093/BIOLRE/IOAE109

Fath-Bayati, L., Naserpour, L., Khoshandam, M., Jannatifar, R., and Fazaeli, H. (2023). Recent advances in developing 3D culture systems of spermatogonial stem cell preservation and differentiation: a narrative review. *Int. J. Reprod. Biomed.* 21, 681–696. doi:10.18502/IJRM.V21I9.14397

Faulk, D. M., Carruthers, C. A., Warner, H. J., Kramer, C. R., Reing, J. E., Zhang, L., et al. (2014). The effect of detergents on the basement membrane complex of a biologic scaffold material. *Acta Biomater.* 10, 183–193. doi:10.1016/j.actbio.2013.09.006

Ghiringhelli, M., Abboud, Y., Chorna, S. V., Huber, I., Arbel, G., Gepstein, A., et al. (2021). Using decellularization/recellularization processes to prepare liver and cardiac engineered tissues. *Methods Mol. Biol.* 2273, 111–129. doi:10.1007/978-1-0716-1246-0_7

Gilpin, A., and Yang, Y. (2017). Decellularization strategies for regenerative medicine: from processing techniques to applications. *Biomed. Res. Int.* 2017, 1–13. doi:10.1155/2017/9831534

Horvath-Pereira, B. de O., Almeida, G. H. D. R., Silva Júnior, L. N. da, do Nascimento, P. G., Horvath Pereira, B. de O., Fireman, J. V. B. T., et al. (2023). Biomaterials for Testicular Bioengineering: how far have we come and where do we have to go? *Front. Endocrinol. (Lausanne)* 14, 1085872. doi:10.3389/fendo.2023.1085872

Kajbafzadeh, A.-M., Khorramirouz, R., Kameli, S. M., Hashemi, J., and Bagheri, A. (2017). Decellularization of human internal mammary artery: biomechanical properties and histopathological evaluation. *Biores Open Access* 6, 74–84. doi:10.1089/biores.2016.0040

Kasinathan, P., Wei, H., Xiang, T., Molina, J. A., Metzger, J., Broek, D., et al. (2015). Acceleration of genetic gain in cattle by reduction of generation interval. *Sci. Rep.* 5, 8674–4. doi:10.1038/srep08674

Kasturi, M., and Vasanthan, K. S. (2023). Effect of decellularization using sodium dodecyl sulfate on glycosaminoglycans content in the liver. *Regen. Med.* 18, 527–530. doi:10.2217/rme-2023-0050

Keane, T. J., Swinehart, I. T., and Badylak, S. F. (2015). Methods of tissue decellularization used for preparation of biologic scaffolds and *in vivo* relevance. *Methods* 84, 25–34. doi:10.1016/j.ymeth.2015.03.005

Khazaei, M. R., Ami, Z., Khazaei, M., and Rezakhani, L. (2023). The decellularized calf testis: introducing suitable scaffolds for spermatogenesis studies. *Int. J. Fertil. Steril.* 18, 32–39. doi:10.22074/IJFS.2023.1989173.1433

Kiani, M., Movahedin, M., Halvaei, I., and Soleimani, M. (2021). Formation of organoid-like structures in the decellularized rat testis. *Iran. J. Basic Med. Sci.* 24, 1523–1528. doi:10.22038/IJBMS.2021.58294.12948

Kulibin, A. Y., and Malolina, E. A. (2023). *In vitro* spermatogenesis: in search of fully defined conditions. *Front. Cell Dev. Biol.* 11, 1106111. doi:10.3389/FCELL.2023.1106111

Laronda, M. M. (2020). Engineering a bioprosthetic ovary for fertility and hormone restoration. *Theriogenology* 150, 8–14. doi:10.1016/j.theriogenology.2020.01.021

Laronda, M. M., Jakus, A. E., Whelan, K. A., Wertheim, J. A., Shah, R. N., and Woodruff, T. K. (2015). Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials* 50, 20–29. doi:10.1016/j.biomaterials.2015.01.051

Lecht, S., Stabler, C. T., Rylander, A. L., Chiaverelli, R., Schulman, E. S., Marcinkiewicz, C., et al. (2014). Enhanced reseeding of decellularized rodent lungs with mouse embryonic stem cells. *Biomaterials* 35, 3252–3262. doi:10.1016/j.biomaterials.2013.12.093

Lee, D. R., Kaproth, M. T., and Parks, J. E. (2001). *In vitro* production of haploid germ cells from fresh or frozen-thawed testicular cells of neonatal bulls 1. *Biol. Reprod.* 65, 873–878. doi:10.1095/biolreprod65.3.873

Lee, H., Han, W., Kim, H., Ha, D.-H., Jang, J., Kim, B. S., et al. (2017). Development of liver decellularized extracellular matrix bioink for three-dimensional cell printing-based liver tissue engineering. *Biomacromolecules* 18, 1229–1237. doi:10.1021/acs.biomac.6b01908

Lynch, C. R., Kondiah, P. P. D., and Choonara, Y. E. (2021). Advanced strategies for tissue engineering in regenerative medicine: a biofabrication and biopolymer perspective. *Molecules* 26, 2518. doi:10.3390/molecules26092518

Moffat, D., Ye, K., and Jin, S. (2022). Decellularization for the retention of tissue niches. *J. Tissue Eng.* 13, 20417314221101151. doi:10.1177/20417314221101151

Morris, A. H., Stamer, D. K., and Kyriakides, T. R. (2017). The host response to naturally-derived extracellular matrix biomaterials. *Semin. Immunol.* 29, 72–91. doi:10.1016/J.SMIM.2017.01.002

Mueller, M. L., and Van Eenennaam, A. L. (2022). Synergistic power of genomic selection, assisted reproductive technologies, and gene editing to drive genetic improvement of cattle. *CABI Agric. Biosci.* 3, 13–29. doi:10.1186/S43170-022-00080-Z

Pasquariello, R., Bogliolo, L., Di Filippo, F., Leoni, G. G., Nieddu, S., Podda, A., et al. (2024). Use of assisted reproductive technologies (ARTs) to shorten the generational interval in ruminants: current status and perspectives. *Theriogenology* 225, 16–32. doi:10.1016/J.THERIOGENOLOGY.2024.05.026

Pennarossa, G., De Iorio, T., Gandolfi, F., and Brevini, T. A. L. (2021a). Ovarian decellularized bioscaffolds provide an optimal microenvironment for cell growth and differentiation *in vitro*. *Cells* 10, 2126. doi:10.3390/CELLS10082126

Pennarossa, G., De Iorio, T., Gandolfi, F., and Brevini, T. A. L. (2022). Impact of aging on the ovarian extracellular matrix and derived 3D scaffolds. *Nanomater. (Basel)* 12, 345. doi:10.3390/NANO12030345

Pennarossa, G., Ghiringhelli, M., Gandolfi, F., and Brevini, T. A. L. (2020). Whole-ovary decellularization generates an effective 3D bioscaffold for ovarian bioengineering. *J. Assist. Reprod. Genet.* 37, 1329–1339. doi:10.1007/s10815-020-01784-9

Pennarossa, G., Ghiringhelli, M., Gandolfi, F., and Brevini, T. A. L. (2021b). Tracheal *in vitro* reconstruction using a decellularized bio-scaffold in combination with a rotating bioreactor. *Methods Mol. Biol.* 2436, 157–165. doi:10.1007/7651_2021_398

Perrard, M. H., Sereni, N., Schluth-Bolard, C., Blondet, A., Giscard d'Estaing, S., Plotton, I., et al. (2016). Complete human and rat *ex vivo* spermatogenesis from fresh or frozen testicular tissue. *Biol. Reprod.* 95, 89–90. doi:10.1095/biolreprod.116.142802

Rajabi-Zeleti, S., Jalili-Firoozinezhad, S., Azarnia, M., Khayyatan, F., Vahdat, S., Nikeghbalian, S., et al. (2014). The behavior of cardiac progenitor cells on macroporous pericardium-derived scaffolds. *Biomaterials* 35, 970–982. doi:10.1016/J.BIOMATERIALS.2013.10.045

Richer, G., Baert, Y., and Goossens, E. (2020). *In-vitro* spermatogenesis through testis modelling: toward the generation of testicular organoids. *Andrology* 8, 879–891. doi:10.1111/ANDR.12741

Salem, M., Khadivi, F., Javanbakht, P., Mojaverrostami, S., Abbasi, M., Feizollahi, N., et al. (2023). Advances of three-dimensional (3D) culture systems for *in vitro* spermatogenesis. *Stem Cell Res. Ther.* 14, 262–330. doi:10.1186/s13287-023-03466-6

Scarrit, M. E., Pashos, N. C., and Bunnell, B. A. (2015). A review of cellularization strategies for tissue engineering of whole organs. *Front. Bioeng. Biotechnol.* 3, 43. doi:10.3389/fbioe.2015.00043

Singh, A., Bivalacqua, T. J., and Sopko, N. (2018). Urinary tissue engineering: challenges and opportunities. *Sex. Med. Rev.* 6, 35–44. doi:10.1016/J.SXMR.2017.08.004

Singh, G., Satpathi, S., Gopala Reddy, B. V., Singh, M. K., Sarangi, S., Behera, P. K., et al. (2023). Impact of various detergent-based immersion and perfusion decellularization strategies on the novel caprine pancreas derived extracellular matrix scaffold. *Front. Bioeng. Biotechnol.* 11, 1253804. doi:10.3389/fbioe.2023.1253804

Sjöqvist, S., Jungebluth, P., Lim, M. L., Haag, J. C., Gustafsson, Y., Lemon, G., et al. (2014). Experimental orthotopic transplantation of a tissue-engineered oesophagus in rats. *Nat. Commun.* 5, 3562. doi:10.1038/ncomms4562

van Maaren, J., Alves, L. F., van Wely, M., van Pelt, A. M. M., and Mulder, C. L. (2023). Favorable culture conditions for spermatogonial propagation in human and non-human primate primary testicular cell cultures: a systematic review and meta-analysis. *Front. Cell Dev. Biol.* 11, 1330830. doi:10.3389/fcell.2023.1330830

Verdile, N., Pasquariello, R., Cardinaletti, G., Tibaldi, E., Brevini, T. A. L., and Gandolfi, F. (2022). Telocytes: active players in the rainbow trout (oncorhynchus mykiss) intestinal stem-cell niche. *Animals* 12, 74. doi:10.3390/ani12010074

Yu, Y. L., Shao, Y. K., Ding, Y. Q., Lin, K. Z., Chen, B., Zhang, H. Z., et al. (2014). Decellularized kidney scaffold-mediated renal regeneration. *Biomaterials* 35, 6822–6828. doi:10.1016/j.biomaterials.2014.04.074

Zhang, J.-K., Du, R.-X., Zhang, L., Li, Y.-N., Zhang, M.-L., Zhao, S., et al. (2017). A new material for tissue engineered vagina reconstruction: acellular porcine vagina matrix. *J. Biomed. Mater Res. A* 105, 1949–1959. doi:10.1002/jbm.a.36066

CHAPTER 5: Isolation and characterization of functional testicular cells

Introduction

Testicular cells engage in dynamic interactions with the extracellular matrix (ECM), sensing and responding to its structural and biochemical cues to regulate spermatogenesis (Siu & Cheng, 2004). To accurately recapitulate this bidirectional communication *in vitro*, both the scaffold described in the previous chapter and the presence of testicular cells are essential to establish a suitable microenvironment for germ cell development. In this context, Sertoli cells and spermatogonia are the primary contributors. Sertoli cells provide structural and metabolic support to developing germ cells, regulate the microenvironment through the secretion of paracrine factors, and form the blood–testis barrier (Hofmann & McBeath, 2022). Spermatogonia, on the other hand, represent the stem cell population of the testis and initiate the spermatogenic cycle (Griswold, 2016).

Given these considerations, the present chapter aims to isolate and characterize specific bovine testicular cell types. In particular, Sertoli cells, spermatogonia-enriched populations, and fibroblasts were separated and characterized using specific markers: SOX9, PGP9.5, and vimentin, respectively.

The cell populations obtained in this study serve as foundamental components for mimicking the native testicular microenvironment and supporting germ cell development *in vitro*.

Biological features and functions of Sertoli and spermatogonial cells

Sertoli cells are highly specialized somatic cells located within the seminiferous tubules of the testis. Often referred to as the "nurse cells" of spermatogenesis, they play a pivotal role in supporting and regulating germ cell development (Xiao et al., 2024). These cells provide essential structural and metabolic support by creating a protected microenvironment conducive to germ cell maturation, supplying nutrients, growth factors, and biochemical signals (Hofmann & McBeath, 2022). Through the formation of tight junctions, Sertoli cells establish the blood–testis barrier, which segregates developing germ cells from the immune system, thereby preventing autoimmune responses (Mruk & Cheng, 2015). Additionally, they exhibit phagocytic activity, removing residual cytoplasm and apoptotic germ cells during spermatogenesis (Ni et al., 2019). Sertoli cells also secrete key regulatory molecules, including inhibin, androgen-binding protein (ABP), and glial cell line-derived neurotrophic factor (GDNF), which modulate testicular function and germ cell differentiation (Chen et al., 2014; Di Persio et al., 2021). Their

responsiveness to gonadotropic hormones such as follicle-stimulating hormone (FSH) and testosterone further underscores their role in orchestrating the testicular microenvironment necessary for successful sperm maturation (Griswold, 1998; Reader et al., 2017; Washburn et al., 2022). Dysregulation of these processes has been associated with impaired fertility and testicular dysfunction (Chen et al., 2014; Reader et al., 2017). Spermatogonia are critical contributors to spermatogenesis, functioning as the earliest pre-meiotic germ cells. They act both as self-renewing stem cells and as progenitors committed to the lifelong production of spermatozoa (Diao et al., 2022; W. Liu et al., 2024). These cells reside along the basement membrane of the seminiferous tubules, where they closely interact with Sertoli cells and the surrounding ECM, forming a specialized microenvironment that regulates their fate through a balance of self-renewal and differentiation cues (W. Liu et al., 2024; Siu & Cheng, 2004). Undifferentiated spermatogonia, including spermatogonial stem cells (SSCs), maintain the germline by undergoing controlled mitotic divisions, while differentiating spermatogonia progressively commit to meiosis and spermiogenesis, ultimately generating mature spermatozoa (Kubota & Brinster, 2018). This dual capacity is tightly regulated by paracrine factors secreted by Sertoli cells, such as Glial cell line-derived neurotrophic factor (GDNF), stem cell factor (SCF/KITL), and

neuregulins, which activate intracellular pathways controlling proliferation, survival, and differentiation (Liu et al., 2024). The localization of spermatogonia in the basal compartment of the seminiferous tubule is critical, as interactions with ECM components (e.g., laminin and collagen IV) and their receptors, particularly β1-integrins, contribute to anchoring the cells within their niche and mediating signal transduction essential for maintaining the blood–testis barrier and the appropriate microenvironment for SSCs (Liu et al., 2024; Siu & Cheng, 2004; Siu & Yan Cheng, 2009). In mammals, spermatogonia are classified into undifferentiated and differentiating subtypes, such as A single, A paired, and A aligned in rodents, or A dark and A pale in primates, reflecting species-specific patterns of mitotic expansion and stem-to-progenitor ratios that influence spermatogenic kinetics (De Rooij & Russell, 2000). Disruption of the spermatogonial population or its somatic support impairs spermatogenesis and leads to infertility, highlighting the fundamental role of this cell population in male reproduction (Leslie et al., 2024; Salonia et al., 2021). Overall, spermatogonia are the cornerstone of germline continuity: through their interactions with Sertoli cells and the ECM, they orchestrate the balance between stemness and differentiation required for normal spermatogenesis, fertility, and the maintenance of testicular homeostasis across species.

Isolation and culture approaches for Sertoli and spermatogonial cells

The isolation of Sertoli cells and spermatogonia from testicular tissue relies on complementary strategies that exploit their distinct biological characteristics. Sertoli cells are typically obtained through sequential enzymatic digestion using collagenase, trypsin, and DNase to dissociate the seminiferous tubules, followed by differential adhesion and selective plating to leverage their strong substrate affinity. Additional purification steps, such as hypotonic shock, are often employed to eliminate residual germ cells, yielding highly viable Sertoli cell populations suitable for *in vitro* applications (Bhushan et al., 2016). In parallel, SSCs can be isolated and enriched via enzymatic digestion combined with marker-based sorting (e.g., c-KIT), and their identity can be functionally validated through xenotransplantation assays (Kubota & Brinster, 2018; Oatley, 2018). Advances in culture systems supplemented with GDNF and leukemia inhibitory factor (LIF) have enabled long-term SSC maintenance and expansion, while optimized cryopreservation protocols preserve their viability and colonization potential (Wang et al., 2014). Moreover, *in vitro* differentiation of spermatogonia has been successfully achieved in cattle and buffalo, underscoring the translational relevance of these

methodologies for fertility preservation, livestock breeding, and germline manipulation (Arain, 2023; Xie et al., 2010).

To recreate a physiologically relevant niche *in vitro*, co-culture of spermatogonia with their somatic counterparts—particularly Sertoli cells—is fundamental, as their reciprocal interactions and paracrine signaling are essential for maintaining stemness, promoting differentiation, and supporting germ cell survival (Hofmann & McBeath, 2022). Current research trends focus on reconstructing a native-like testicular microenvironment by integrating Sertoli cells, SSCs, and ECM components (Alves-Lopes & Stukenborg, 2018). Such platforms hold great promise for elucidating cell–cell and cell–matrix signaling mechanisms, assessing the effects of toxicants and endocrine disruptors, and developing SSC-based biotechnologies for genetic improvement and fertility preservation in cattle (Cortez et al., 2022).

Materials and methods

Ethic statement

Bovine testes were collected from four 2-year-old bulls at a local abattoir. As the organs were obtained from animals slaughtered for human consumption, their use did not constitute animal experimentation under

Directive 2010/63/EU of the European Parliament. All procedures were conducted in accordance with approved ethical guidelines.

Samples collection

Once collected, testes were transported to the laboratory in cold sterile saline solution (0.9% NaCl) within one hour. Upon arrival, the organs were washed with Dulbecco phosphate-buffered saline (DPBS) and maintained on ice until further processing.

Isolation of Sertoli and fibroblasts cells

Testicular cell lines were established in-house. Following dissection, fragments of testicular parenchyma (~2 mm^3) were washed three times in sterile DPBS supplemented with 2% antibiotic/antimycotic solution (A5955-100ml, Sigma-Aldrich) and placed in 33 × 10 mm Petri dishes (Sarstedt, Nümbrecht, Germany) pre-coated with 0.1% pig skin-derived gelatin. Droplets of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 20% fetal calf serum (FCS) and 2% antibiotic/antimycotic solution were gradually added to cover the tissue fragments. Cultures were incubated at 37 °C with 5% CO_2 for two weeks until cell outgrowth was observed. Tissue fragments were then removed, and adherent cells were detached using Trypsin-EDTA and

transferred to 25 cm^2 culture flasks. To enrich specific cell populations, two rounds of differential plating were performed, each with an incubation time of approximately one hour. This procedure enabled the isolation of fibroblasts and Sertoli cells, which were subsequently cultured in DMEM/F12 supplemented with 5% FBS at 37 °C with 5% CO_2 and passaged twice weekly at a 1:3 ratio. Proliferative performance was monitored by recording split ratios and time-to-confluence at each passage, and by evaluating post-thaw recovery at passage 20.

An aliquot of these cells was used for immunocytochemistry analyses.

Isolation and purification of spermatogonia

After decapsulation, the testicular parenchyma was finely minced into small pieces using a scalpel. The tissue fragments were enzymatically digested in 20 mL of DMEM/F12 (Thermo Fisher Scientific) containing 1 mg/mL collagenase IV (Gibco) and 1 mg/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 30 minutes. The resulting cell suspension was centrifuged at 500 × g for 5 minutes at room temperature and washed at least twice with DPBS. The pellet was resuspended in 10 mL of DMEM/F12 and filtered sequentially through 70 μm and 40 μm nylon mesh strainers. After centrifugation at 250 × g for 2 minutes at room temperature, the pellet was resuspended in 0.5 mL of

DMEM/F12 and layered onto a discontinuous Percoll gradient (1 mL each of 90%, 65%, 50%, 40%, 36%, 34%, 32%, 30%, 28%, and 20% Percoll in DMEM/F12) in a 15 mL conical tube, following a modified version of the protocol described by (Izadyar, 2002). The tube was centrifuged at 650 × g for 25 minutes. Cells located in the interphase between the 30% and 36% Percoll layers were collected, washed with warm DPBS, and resuspended in 1 mL of DMEM/F12 culture medium. The suspension was filtered through a 15 μm nylon mesh strainer. A portion of the isolated cells was processed for immunocytochemistry.

Marker validation and immunohistochemistry analyses

Antibodies against vimentin, SOX9, and PGP9.5 were validated in bovine testis according to the manufacturers' protocols. Briefly, 0.5 cm^3 pieces of testicular parenchyma were fixed in 10% neutral buffered formalin for 48 hours at room temperature, dehydrated in graded alcohols, cleared with xylene, and embedded in paraffin. Sections (5 μm thick) were cut, dewaxed, rehydrated, and subjected to antigen retrieval in 10 mM sodium citrate buffer with 0.05% Tween-20 (pH 6) at 97–99 °C for 25 minutes. After cooling in distilled water at room temperature for 20 minutes, sections were blocked with 10% goat serum for 30 minutes. Primary antibodies (SOX9, PGP9.5, and vimentin) were applied at 1:250 dilution

in 4% bovine serum albumin (BSA) and incubated for 1 hour at room temperature in a humidified chamber. Slides were then incubated with appropriate secondary antibodies (1:250) for 30 minutes at room temperature, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and mounted using ProLong™ Gold Antifade Mountant (ThermoFisher Scientific, Waltham, MA, USA). Slides were air-dried overnight and examined under a microscope.

Immunostaining conditions were optimized separately for ICC/IF (cultured cells) and IHC/IF on formalin-fixed, paraffin-embedded (FFPE) tissue sections. Although the same primary antibody was used in both applications, monolayer cultures and FFPE sections differ substantially in epitope accessibility, diffusion/penetration constraints, and background sources due to differences in sample thickness, fixation/processing, and matrix complexity. In FFPE tissue, crosslinking fixation and paraffin embedding can further reduce epitope availability, requiring section-specific optimization.

For ICC/IF, the primary antibody was incubated overnight at 4 °C to maximize specific binding while limiting nonspecific interactions and improving the signal-to-noise ratio in monolayer preparations; secondary antibody incubation was performed under standard conditions at room temperature.

For IHC/IF on FFPE sections, primary antibody incubation time and temperature were selected based on optimization/validation on sectioned material and aligned with standard FFPE tissue-staining workflows, to obtain optimal specific signal with low background while accounting for tissue architecture and antibody penetration. Importantly, the ICC/IF protocol had been previously validated in our laboratory for this cellular model and was maintained unchanged to ensure reproducibility and comparability across experiments.

Characterization of isolated cells and immunocytochemistry analyses

Fibroblasts and Sertoli cells were seeded in 24-well plates and cultured until reaching 70–80% confluency, then fixed in cold methanol for 30 minutes at room temperature. To block non-specific binding, cells were incubated with 10% goat serum for 30 minutes. Fibroblasts were incubated overnight at 4 °C with anti-vimentin antibody (1:250, Abcam, ab92547), and Sertoli cells with anti-SOX9 antibody (1:250, Merck Millipore, AB5535). The following day, fibroblasts were incubated with Alexa Fluor™ 488 goat anti-rabbit secondary antibody (Life Technologies Corporation, A27034, Carlsbad, CA, USA), and Sertoli cells with Alexa Fluor™ 594 goat anti-rabbit (Life Technologies Corporation, A11012, Carlsbad, CA, USA) for 30 minutes at room temperature in the dark. After

four PBS washes, DAPI was applied for 15 minutes to counterstain nuclei. Images were acquired using an Eclipse TE200 microscope (Nikon, Tokyo, Japan).

Isolated spermatogonia were fixed in 4% paraformaldehyde for 30 minutes at room temperature, centrifuged at 98.4 × g for 5 minutes, and washed twice with PBS. Cells were transferred onto glass slides using a cytocentrifuge at 450 rpm for 5 minutes and air-dried. They were then fixed and permeabilized in cold methanol for 10 minutes in a humidified chamber. After blocking with 10% goat serum for 30 minutes, cells were incubated with anti-PGP9.5 antibody (1:250, Abcam, ab8189) for 1 hour at room temperature, washed three times with PBS, and incubated with Alexa Fluor™ 594 goat anti-mouse secondary antibody (Life Technologies Corporation, A11058, Willow Creek Road, OG, USA). Slides were counterstained with DAPI for 15 minutes and mounted with ProLong™ Gold Antifade Mountant. Imaging was performed using an Eclipse E600 microscope (Nikon, Amsterdam, Netherlands) equipped with a digital camera. Images were acquired using NIS-Elements Software (Version 4.6; Nikon).

Cells counting

The number of SOX9- and vimentin-positive cells was quantified in 15 randomly selected fields at 200× magnification. A minimum of 600 cells were counted across three independent replicates. Results were expressed as the percentage of positively stained cells relative to the total number of cells counted.

Results

Isolation of Sertoli and fibroblasts cells

After 10 days of culture, cells began to grow out from the tissue fragments and proliferated rapidly. Once confluency was reached, the fragments were carefully removed. Initially, the culture displayed heterogeneous morphology, including fibroblast-like and epithelial-like cells (Figure 1).

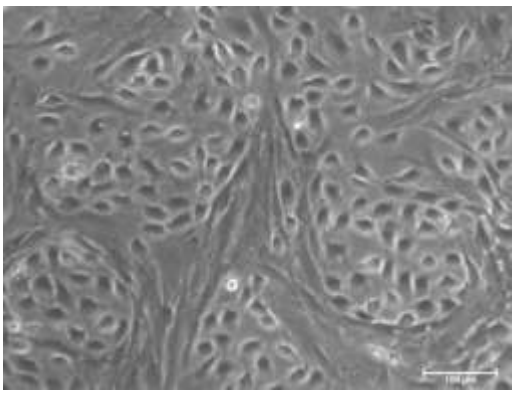

Figure 1. Heterogeneous Culture of Fibroblast and Epithelial-like Cells. *Mixed population of fibroblast-like and epithelial-like cells showing heterogeneous distribution across the culture surface.*

Following two rounds of one-hour differential plating, two distinct cell populations were observed: one exhibiting morphological features consistent with Sertoli cells, and the other resembling fibroblasts (Figure 2).

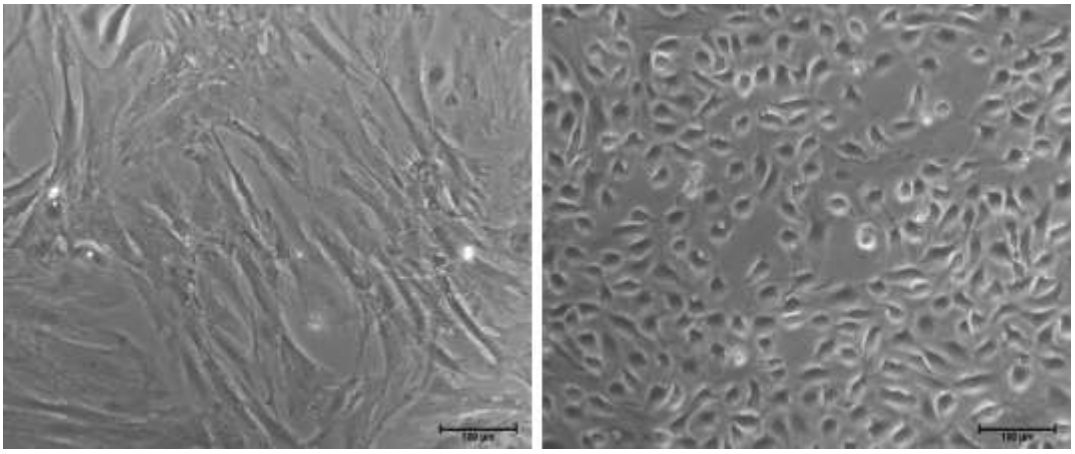

Figure 2. Representative images of isolated cells. *Morphologically distinct cell types observed after isolation. The left panel shows fibroblast-like cells, characterized by a spindle-shaped appearance, elongated nuclei, and abundant cytoplasm. The right panel displays epithelial-like cells, identified by their polygonal shape, round nuclei, and reduced cytoplasmic volume.*

Isolation of spermatogonia

Following enzymatic digestion and filtration of the testicular cell suspension, microscopic examination revealed that most collected cells exhibited morphological features characteristic of undifferentiated spermatogonia. These included a relatively large, centrally located round or slightly oval nucleus with a prominent nucleolus, easily visible under phase-contrast microscopy. The cytoplasm appeared moderate and finely

granular, with a high nucleus-to-cytoplasm ratio and clearly defined cell borders (Figure 3).

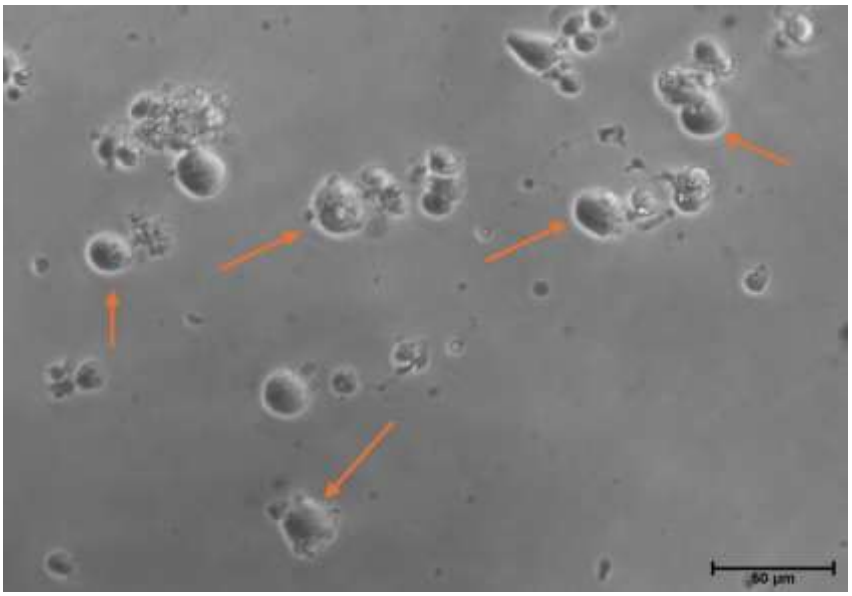

Figure 3. Representative image of isolated spermatogonia *Isolated spermatogonia appear as small, round cells with a high nucleus-to-cytoplasm ratio and minimal cytoplasmic extensions, consistent with their undifferentiated morphology.*

Immunohistochemistry analyses and marker validation

Sertoli-like cells were identified using SOX9 and vimentin, validated on native testicular tissue sections. SOX9, a nuclear marker, was specifically expressed in Sertoli cells located in the basal compartment of the seminiferous tubules, showing distinct nuclear localization. Vimentin exhibited cytoplasmic distribution within Sertoli cells and was also detected in the interstitial space, where fibroblasts are predominantly located (Figure 4).

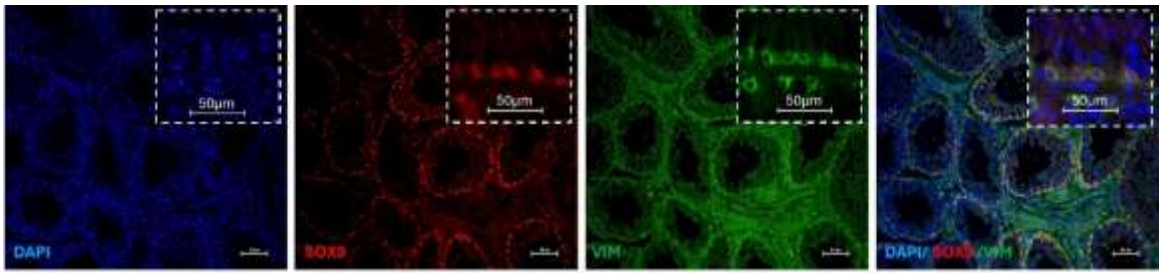

Figure 4. Immunofluorescence staining of bovine testicular tissue. *SOX9 shows strong nuclear localization in Sertoli cells, whereas vimentin is distributed throughout the cytoplasm surrounding the seminiferous tubules. Nuclei were counterstained with DAPI (blue).*

To identify germ cells, PGP9.5 was used and similarly validated on native tissue. PGP9.5 showed cytoplasmic localization in cells within the basal compartment of the seminiferous tubules, the typical niche of spermatogonia (Figure 5).

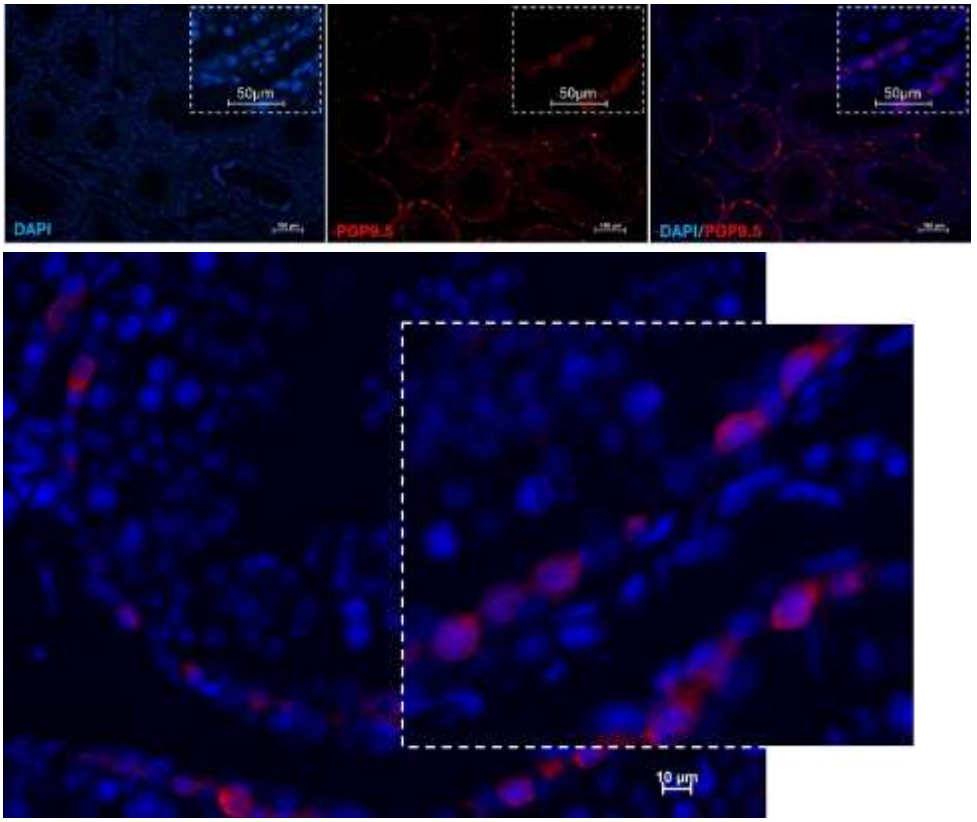

Figure 5. Immunohistochemistry of the native tissue. *Immunohistochemical detection of PGP9.5 in native testicular tissue. Panel A shows a low-magnification view of the seminiferous tubules, while panel B presents a higher magnification. Positive cytoplasmic staining for PGP9.5 (red) was observed in cells located along the basal compartment of the seminiferous epithelium, consistent with the typical localization of spermatogonia.*

Identification of testicular cell types

After validation on native tissue, isolated cells were subjected to immunocytochemistry. SOX9 was clearly localized in the nuclei of Sertoli-like cells, while vimentin was confined to the cytoplasm. Nuclei were counterstained with DAPI (Figure 6A). In contrast, fibroblast-like cells were negative for SOX9 but showed cytoplasmic positivity for vimentin (Figure 6B). Quantitative analysis revealed that 88.24% of the

isolated epithelial-like cells were SOX9-positive Sertoli cells, while 99% of the fibroblast-like cells were confirmed as fibroblasts (Figure 6C).

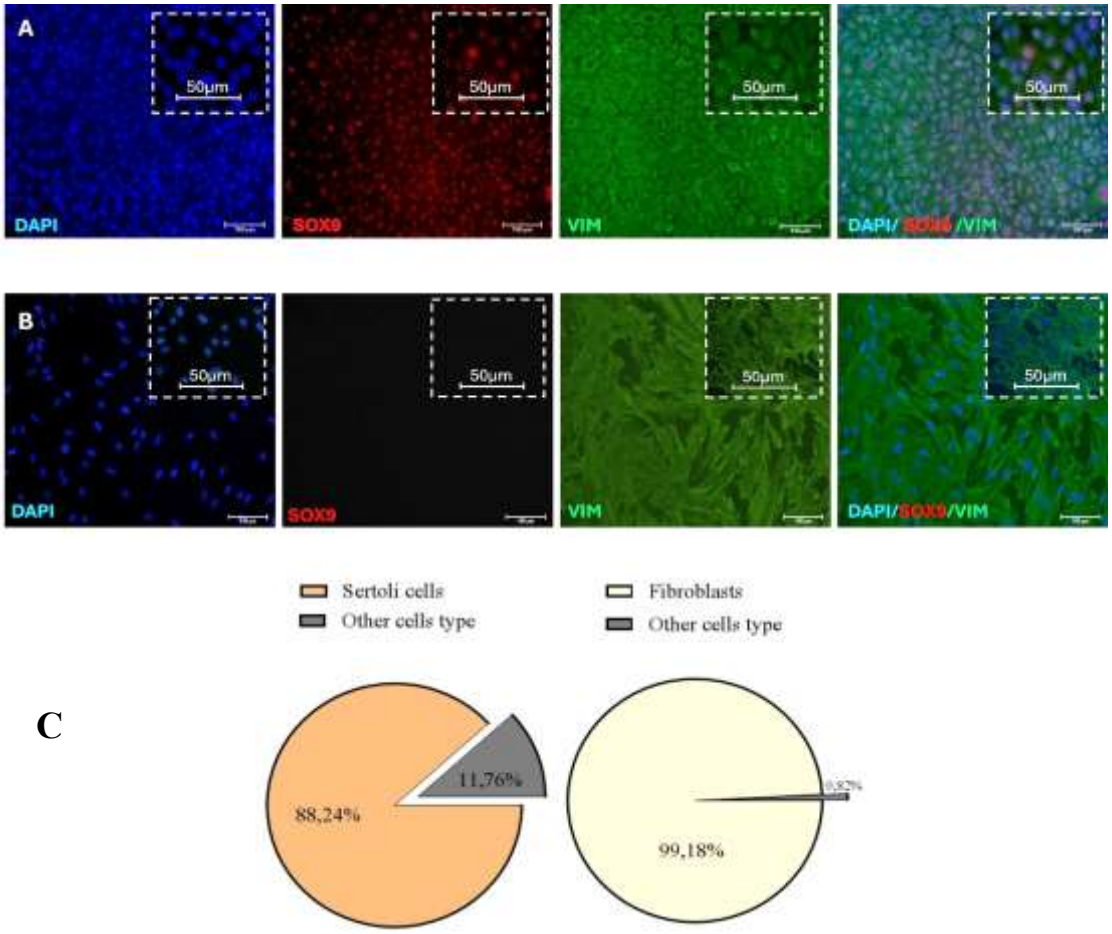

Figure 6. Immunofluorescence characterization of Sertoli and fibroblast-like cells and quantification of Sertoli cell purity. *(A) Epithelial-like cells were identified as Sertoli cells based on co-expression of SOX9 (red, nuclear) and vimentin (green, cytoplasmic), with DAPI (blue) nuclear counterstaining. (B) Fibroblast-like cells were negative for SOX9 but positive for vimentin. (C) Quantification of isolated Sertoli cells based on SOX9 immunopositivity.*

Long-term expansion and phenotypic maintenance.

Proliferative performance was assessed using longitudinal culture kinetics rather than dedicated cell-cycle markers like ki67. Across serial passaging, cells showed reproducible expansion, as indicated by consistent split ratios and comparable seeding-to-confluence intervals. Cultures were routinely passaged at a maximum split ratio of 1:4 approximately every 3 days, including under reduced serum conditions (5% FCS), suggesting sustained expansion competence. At passage 20, cells were cryopreserved and subsequently thawed; post-thaw cultures rapidly re-attached and regained confluence with kinetics comparable to pre-freeze cultures, supporting preserved recovery and expansion capacity after cryostorage. Phenotypic identity at late passage was assessed by SOX9 immunostaining performed at passage 20 both prior to and after cryopreservation, showing a clear nuclear signal consistent with maintenance of cell identity. Representative images are not available.

Identification of spermatogonia

Although morphological features suggested the presence of spermatogonia, their identity was further confirmed by immunofluorescence analysis using PGP9.5, a well-established marker of undifferentiated bovine germ cells (Luo, 2006) (Figure 7).

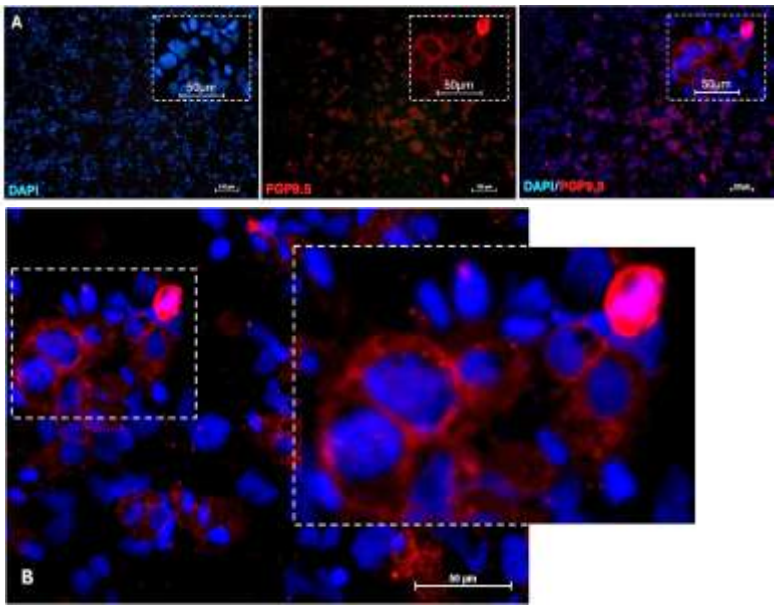

***Figure 7. Immunocytochemical analysis of isolated* Spermatogonia**. *Panel A shows lower magnification of the culture, while panel B shows higher magnification. Spermatogonia exhibited cytoplasmic PGP9.5 staining (red), confirming their identity. Nuclei were counterstained with DAPI (blue).*

Discussion

In this study, we successfully isolated three main cell populations from bovine testes: Sertoli cells, fibroblasts, and an enriched fraction of spermatogonia. Sertoli cells are essential epithelial cells that support germ cells and are critical for maintaining the spermatogonial niche and

regulating spermatogenesis (Griswold, 1998; Oatley & Brinster, 2008). Fibroblasts, derived from mesenchymal tissue, contribute to the structural integrity of the testicular environment and secrete signaling molecules that influence the testicular niche (Shen et al., 2021). Spermatogonia, the progenitors of spermatozoa, play a pivotal role in male fertility by ensuring a continuous supply of gametes through a tightly regulated differentiation process.

While fibroblasts are relatively easy to isolate and culture, the derivation of Sertoli cells remains more challenging. Although Sertoli cell lines are less common, several studies have reported successful derivation from mammalian species, and ongoing research continues to improve their isolation and stability. For example, bovine Sertoli cells have been successfully cultured and show promise for applications in reproductive biology (Bernardino et al., 2018). However, maintaining their proliferative capacity and functional phenotype over extended culture periods remains a significant challenge (Saewu et al., 2020).

In our study, Sertoli cells were derived from testicular tissue explants and purified via differential plating, resulting in an epithelial-like subculture. The purity of the culture was assessed by quantifying SOX9-positive cells, a well-established nuclear marker for Sertoli cells, previously validated in mouse (Hemendinger et al., 2002), human (Chui et al., 2011), and dog

(Banco et al., 2010). Vimentin, a cytoskeletal protein, was also used as a marker, although it is expressed in other cell types such as fibroblasts and spermatogonia (Zomer & Reddi, 2020). Despite this, its cytoplasmic localization provides valuable information on cellular morphology and organization. The combined use of SOX9 and vimentin offers a robust strategy for distinguishing Sertoli cells from other testicular cell types.

These markers were also validated in vivo using formalin-fixed, paraffin-embedded bovine testis sections. Cells located in the basal compartment of the seminiferous tubules, extending toward the lumen—consistent with Sertoli cell anatomy—exhibited nuclear SOX9 and cytoplasmic vimentin staining, supporting their identification.

The purity of our Sertoli cell preparations ranged from 85% to 90%, aligning with previous reports across species. For instance, mouse primary Sertoli cultures often exceed 90% purity following enzymatic isolation and lineage-based sorting (Saewu et al., 2020). In rats, enzymatic digestion typically yields ~80% purity, with residual contamination from germ and peritubular myoid cells (Anway et al., 2003). The highest purity has been reported in human models, where advanced techniques such as flow cytometry and lectin-mediated adhesion can achieve >97% purity (Lakpour et al., 2017). We grew the isolated Sertoli cells in 25 cm^2 culture flasks with DMEM/F12. Cells reached confluence every 3 days and were

passaged at a 1:4 Ratio. A limitation of this study is that proliferation was not quantified using dedicated proliferation/cell-cycle assays (e.g., Ki67 immunostaining, EdU/BrdU incorporation, or population doubling time calculations). Instead, proliferative competence was inferred from longitudinal culture kinetics (split ratio and time-to-confluence), as well as from preserved post-thaw recovery at passage 20. In addition, SOX9 staining at passage 20 was not documented with representative images. Future work will include marker-based and quantitative assays to further substantiate long-term proliferative capacity and exclude senescence-associated changes.

Differential plating also enabled the establishment of a mesenchymal cell line with spindle-shaped morphology, consistent with fibroblasts. Their identity was confirmed by cytoplasmic vimentin expression and absence of nuclear SOX9 staining. Immunohistochemistry on native tissue confirmed the typical spatial distribution of fibroblasts within the interstitial compartment, further validating marker specificity.

The spermatogonia-enriched fraction, obtained via Percoll gradient centrifugation, was identified based on morphological criteria: small (10–12 μm), round cells with a high nuclear-to-cytoplasmic ratio and refractile cytoplasm, often forming tightly packed clusters. These features are consistent with previous descriptions of cattle (Aponte, 2020) and goat

(Heidari et al., 2012a; Singh et al., 2022) spermatogonia isolation protocols. In this study, testes were obtained from adult bulls (2 years old), where spermatogenic tissue contains a broad spectrum of germ cell stages and abundant somatic cells. We therefore adopted discontinuous Percoll density gradient centrifugation as an initial enrichment step because it is a cost-effective, antibody-independent method that can be applied to large cell numbers and that efficiently reduces debris and cellular aggregates prior to downstream analyses.

Importantly, Percoll-based enrichment has been previously used in bovine spermatogonial studies and can yield substantial enrichment of type A spermatogonia when combined with additional purification steps.

To confirm their identity, immunostaining for PGP9.5 (also known as UCHL-1), a conserved cytoplasmic marker of undifferentiated spermatogonia, was performed. PGP9.5 has been reported to label undifferentited spermatogonia in bovine testes and can be applied to isolated testicular cell preparations (Goel et al., 2011). Because the aim of this study was to enrich and identify the spermatogonial compartment rather than to resolve spermatogonial sub-states, multi-marker panels were not implemented. Future work will incorporate additional markers (e.g., PLZF together with c-KIT) to refine subtype discrimination.

This marker has been validated in multiple species, including pig (Luo et al., 2006), goat (Heidari et al., 2012b), donkey (Choi et al., 2020), and bovine (Herrid et al., 2007). In our samples, PGP9.5 showed cytoplasmic localization in cells situated in the basal compartment of the seminiferous tubules, consistent with the expected position of germline stem cells.

Conclusions

This study presents our initial efforts to establish a robust protocol for the isolation and characterization of the two testicular cell types—Sertoli cells and spermatogonia—that play a central role in spermatogenesis. The approach lays the groundwork for the next phase of the project, which will involve repopulating decellularized testicular bioscaffolds. This will allow us to investigate their ability to support the establishment of functional interactions between the two cell types and to promote progression toward meiotic division.

*CHAPTER 6: **Overall conclusions***

In this Ph.D. thesis, an integrated approach was developed to address two major challenges in bovine reproductive biotechnology: the use of spermatids as an alternative to mature spermatozoa, and the establishment of the two key components of the testicular microenvironment, namely a three-dimensional scaffold mimicking the tubular architecture and primary testicular cell populations that drive germ cell development.

The first part of this thesis focused on establishing a protocol for the isolation, identification, and quality assessment of bovine spermatids, followed by evaluation of their fertilizing capacity through injection into *in in vitro*–matured oocytes. Although fertilization rates were low, this represents an important initial step toward the use of immature male gametes to accelerate genetic progress in livestock.

The second part of this work focused on the two main components of the testicular niche: the acellular compartment (extracellular matrix and tubular structure) and the cellular counterpart. On one hand, it involved the development and validation of a protocol for generating decellularized bovine testicular scaffolds that preserve the native architecture and key extracellular matrix (ECM) components. These scaffolds were subsequently repopulated with testicular fibroblasts, demonstrating their biocompatibility, which is a critical prerequisite for the creation of more

advanced 3D models. On the other hand, this study successfully isolated and characterized Sertoli cells, spermatogonia-enriched populations, and testicular fibroblasts using specific markers (SOX9, PGP9.5, and vimentin). The establishment of standardized isolation protocols ensures the availability of well-defined cell populations, which are indispensable for reconstructing the complexity of the testicular microenvironment.

Overall, this work established protocols for a few key steps toward the ultimate goal of generating male haploid cells suitable for injection into mature oocytes. Although the task confirmed to be highly complex, the gradual and systematic approach adopted in this study contributes to laying the groundwork for future advancements in the field.

CHAPTER 7:
Acknowledgements

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CHAPTER 8: ***Bibliography***

Alves-Lopes, J. P., & Stukenborg, J. B. (2018). Testicular organoids: a new model to study the testicular microenvironment in vitro? *Human Reproduction Update*, *24*(2), 176–191. https://doi.org/10.1093/HUMUPD/DMX036

Arain, S. (2023). Biotechnological therapies for animal reproduction in the livestock sector. *Pure and Applied Biology*, *12*(2). https://doi.org/10.19045/bspab.2023.120130

Arya, A., Trivedi, M. M., Modi, R. J., & Patel, Y. G. (2024). A Look at Genomic Selection Techniques for Climate Change Adaptation and Production in Livestock. *Journal of Scientific Research and Reports*, *30*(6), 427–436. https://doi.org/10.9734/JSRR/2024/V30I62059

Asem-Hiablie, S., Battagliese, T., Stackhouse-Lawson, K. R., & Alan Rotz, C. (2019). A life cycle assessment of the environmental impacts of a beef system in the USA. *International Journal of Life Cycle Assessment*, *24*(3), 441–455. https://doi.org/10.1007/S11367-018-1464-6/FIGURES/4

Aussillous, P., & Quéré, D. (2001). Liquid marbles. *Nature*, *411*(6840), 924–927. https://doi.org/10.1038/35082026

Bagath, M., Krishnan, G., Devaraj, C., Rashamol, V. P., Pragna, P., Lees, A. M., & Sejian, V. (2019). The impact of heat stress on the immune system in dairy cattle: A review. *Research in Veterinary Science*, *126*, 94–102. https://doi.org/10.1016/J.RVSC.2019.08.011

Barth, A., Perry, V. E. A., Hamilton, L. E., Sutovsky, P., & Oko, R. (2025). Bovine Spermatogenesis. *Advances in Anatomy, Embryology, and Cell Biology*, *240*, 65–136. https://doi.org/10.1007/978-3-031-70126-9_2

Baruselli, P. S., de Carvalho, N. A. T., Gasparrini, B., Campanile, G., & D'Occhio, M. J. (2023). Review: Development, adoption, and impact of assisted reproduction in domestic buffaloes. *Animal*, *17*, 100764. https://doi.org/10.1016/J.ANIMAL.2023.100764

Bashiru, H. A., & Oseni, S. O. (2025). Simplified climate change adaptation strategies for livestock development in low-and middle-income countries. *Frontiers in Sustainable Food Systems*, *9*, 1566194. https://doi.org/10.3389/FSUFS.2025.1566194/FULL

Berglund, B. (2008). Genetic Improvement of Dairy Cow Reproductive Performance. *Reproduction in Domestic Animals*, *43*(SUPPL.2), 89–95. https://doi.org/10.1111/J.1439-0531.2008.01147.X

Bernabucci, U., Biffani, S., Buggiotti, L., Vitali, A., Lacetera, N., & Nardone, A. (2014). The effects of heat stress in Italian Holstein dairy cattle. *Journal of Dairy Science*, *97*(1), 471–486. https://doi.org/10.3168/JDS.2013-6611

Bett, B., Kiunga, P., Gachohi, J., Sindato, C., Mbotha, D., Robinson, T., Lindahl, J., & Grace, D. (2017). Effects of climate change on the occurrence and distribution of livestock diseases. *Preventive Veterinary Medicine*, *137*(Pt B), 119–129. https://doi.org/10.1016/J.PREVETMED.2016.11.019

Bhushan, S., Aslani, F., Zhang, Z., Sebastian, T., Elsässer, H. P., & Klug, J. (2016). Isolation of Sertoli Cells and Peritubular Cells from Rat Testes. *Journal of Visualized Experiments : JoVE*, *2016*(108), 53389. https://doi.org/10.3791/53389

Bijma, P., & Woolliams, J. A. (1999). Prediction of genetic contributions and generation intervals in populations with overlapping generations under selection. *Genetics*, *151*(3), 1197. https://doi.org/10.1093/GENETICS/151.3.1197

Bishop, T. F., & Van Eenennaam, A. L. (2020). Genome editing approaches to augment livestock breeding programs. *The Journal of Experimental Biology*, *223*(Pt Suppl 1). https://doi.org/10.1242/JEB.207159

Brevini, T. A. L., Pennarossa, G., & Gandolfi, F. (2020). A 3D approach to reproduction. *Elsevier*, *150*, 2–7. https://doi.org/10.1016/J.THERIOGENOLOGY.2020.01.020

Brocke, S. A., Speen, A. M., Masood, S., Worden, C. P., & Jaspers, I. (2024). Comparison of permeable cell culture inserts for use in culture of a human in vitro air–liquid interface model system. *Physiological Reports*, *12*(3). https://doi.org/10.14814/phy2.15921

Chen, L. Y., Brown, P. R., Willis, W. B., & Eddy, E. M. (2014). Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. *Endocrinology*, *155*(12), 4964–4974. https://doi.org/10.1210/en.2014-1406

Chen, Y. S., & Qiu, X. B. (2012). Transcription-coupled replacement of histones: Degradation or recycling? In *Journal of Genetics and Genomics* (Vol. 39, Issue 11, pp. 575–580). https://doi.org/10.1016/j.jgg.2012.09.001

Cortez, J., Leiva, B., Torres, C. G., Parraguez, V. H., De los Reyes, M., Carrasco, A., & Peralta, O. A. (2022). Generation and Characterization of Bovine Testicular Organoids Derived from Primary Somatic Cell Populations. *Animals 2022, Vol. 12, Page 2283*, *12*(17), 2283. https://doi.org/10.3390/ANI12172283

Costello, L., Darling, N., Freer, M., Bradbury, S., Mobbs, C., & Przyborski, S. (2021). Use of porous polystyrene scaffolds to bioengineer human epithelial tissues in vitro. *SpringerL Costello, N Darling, M Freer, S Bradbury, C Mobbs, S PrzyborskiNext Generation Culture Platforms for Reliable In Vitro Models: Methods and, 2021•Springer*, *2273*, 279–296. https://doi.org/10.1007/978-1-0716-1246-0_20

De Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. In *Journal of Andrology* (Vol. 21, Issue 6, pp. 776–798). https://doi.org/10.1002/j.1939-4640.2000.tb03408.x

Decker, J. (2021). Decreasing generation interval to increase genetic progress. *Harvested from the MU Extension Website, May 2021.* https://mospace.umsystem.edu/xmlui/handle/10355/85172

Di Filippo, F., Brevini, T. A. L., Pennarossa, G., & Gandolfi, F. (2024). Generation of bovine decellularized testicular bio-scaffolds as a 3D platform for testis bioengineering. *Frontiers in Bioengineering and Biotechnology*, *12*. https://doi.org/10.3389/fbioe.2024.1532107

Di Persio, S., Starace, D., Capponi, C., Saracino, R., Fera, S., Filippini, A., & Vicini, E. (2021). TNF-α inhibits GDNF levels in Sertoli cells, through a NF-κB-dependent, HES1-dependent mechanism. *Andrology*, *9*(3), 956–964. https://doi.org/10.1111/andr.12959

Diao, L., Turek, P. J., John, C. M., Fang, F., & Reijo Pera, R. A. (2022). Roles of Spermatogonial Stem Cells in Spermatogenesis and Fertility Restoration. *Frontiers in Endocrinology*, *13*, 895528. https://doi.org/10.3389/FENDO.2022.895528/BIBTEX

Díez, M., Przyborski, S., del Cerro, A., Alonso-Guervós, M., Iglesias-Cabo, T., Carrocera, S., García, M., Fernández, M., Alonso, L., & Muñoz, M. (2023). Generation of a novel three-dimensional scaffold-based model of the bovine endometrium. *Veterinary Research Communications*, *47*(3), 1721. https://doi.org/10.1007/S11259-023-10130-0

Eastwood, C. R., Edwards, J. P., & Turner, J. A. (2021). Review: Anticipating alternative trajectories for responsible Agriculture 4.0 innovation in livestock systems. *Animal*, *15*. https://doi.org/10.1016/j.animal.2021.100296

Emmerling, C., Krein, A., & Junk, J. (2020). Meta-Analysis of Strategies to Reduce NH3 Emissions from Slurries in European Agriculture and Consequences for Greenhouse Gas Emissions. *Agronomy 2020, Vol. 10, Page 1633*, *10*(11), 1633. https://doi.org/10.3390/AGRONOMY10111633

FAO. (2017). *FAO (2017)*. https://www.scirp.org/reference/referencespapers?referenceid=3381401

FAO. (2023). *FAO 2023*. https://www.fao.org/events/detail/fao-global-conference-on-sustainable-livestock-transformation-2023/en

Frattini, S., Capra, E., Lazzari, B., McKay, S. D., Coizet, B., Talenti, A., Groppetti, D., Riccaboni, P., Pecile, A., Chessa, S., Castiglioni, B., Williams, J. L., Pagnacco, G., Stella, A., & Crepaldi, P. (2017). Genome-wide analysis of DNA methylation in hypothalamus and ovary of Capra hircus. *BMC Genomics*, *18*(1). https://doi.org/10.1186/s12864-017-3866-4

Friggens, N. C., Blanc, F., Berry, D. P., & Puillet, L. (2017). Review: Deciphering animal robustness. A synthesis to facilitate its use in livestock breeding and management. *Animal*, *11*(12), 2237–2251. https://doi.org/10.1017/S175173111700088X

García-Ruiz, A., Cole, J. B., VanRaden, P. M., Wiggans, G. R., Ruiz-López, F. J., & Van Tassell, C. P. (2016). Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(28), E3995–E4004. https://doi.org/10.1073/PNAS.1519061113

Godde, C. M., Mason-D'Croz, D., Mayberry, D. E., Thornton, P. K., & Herrero, M. (2021). Impacts of climate change on the livestock food supply chain; a review of the evidence. *Global Food Security*, *28*, 100488. https://doi.org/10.1016/J.GFS.2020.100488

Goel, S., Mahla, R. S., Suman, S. K., Reddy, N., & Imai, H. (2011). UCHL-1 protein expression specifically marks spermatogonia in wild bovid testis. *European Journal of Wildlife Research*, *57*(3), 663–667. https://doi.org/10.1007/s10344-010-0454-1

Grant, L., Vanderkelen, I., Gudmundsson, L., Fischer, E., Seneviratne, S. I., & Thiery, W. (2025). Global emergence of unprecedented lifetime exposure to climate extremes. *Nature*, *641*(8062), 374. https://doi.org/10.1038/S41586-025-08907-1

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. In *CELL & DEV ELOPMENTAL BI OLOGY* (Vol. 9).

Griswold, M. D. (2016). Spermatogenesis: The Commitment to Meiosis. *Physiol Rev*, *96*, 1–17. https://doi.org/10.1152/physrev.00013.2015.-Mam

Hayes, B. J., Bowman, P. J., Chamberlain, A. J., & Goddard, M. E. (2009). Invited review: Genomic selection in dairy cattle: Progress and challenges. *Journal of Dairy Science*, *92*(2), 433–443. https://doi.org/10.3168/JDS.2008-1646

Hocquette, J. F., Lehnert, S., Barendse, W., Cassar-Malek, I., & Picard, B. (2007). Recent advances in cattle functional genomics and their application to beef quality. *Animal*, *1*(1), 159–173. https://doi.org/10.1017/S1751731107658042

Hofmann, M. C., & McBeath, E. (2022). Sertoli Cell-Germ Cell Interactions Within the Niche: Paracrine and Juxtacrine Molecular Communications. *Frontiers in Endocrinology*, *13*, 897062. https://doi.org/10.3389/FENDO.2022.897062/FULL

Hong, C., Zhong, R., Xu, M., He, P., Mo, H., Qin, Y., Shi, D., Chen, X., He, K., & Zhang, Q. (2025). Interactions Among Food Systems, Climate Change, and Air Pollution: A Review. *Engineering*, *44*, 215–233. https://doi.org/10.1016/J.ENG.2024.12.021

Ishiguro, K. ichiro. (2023). Mechanism of initiation of meiosis in mouse germ cells. *Current Topics in Developmental Biology*, *151*, 1–26. https://doi.org/10.1016/BS.CTDB.2022.04.005

Izadyar, F. , S. G. T. , C. L. B. , den O. K. , & de R. D. G. (2002). Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction*, 85. – 94.

Kasimanickam, R., Ferreira, J. C. P., Kastelic, J., & Kasimanickam, V. (2025). Application of Genomic Selection in Beef Cattle Disease Prevention. *Animals 2025, Vol. 15, Page 277*, *15*(2), 277. https://doi.org/10.3390/ANI15020277

Khan, I., Mesalam, A., Heo, Y. S., Lee, S. H., Nabi, G., & Kong, I. K. (2023). Heat Stress as a Barrier to Successful Reproduction and

Potential Alleviation Strategies in Cattle. *Animals 2023, Vol. 13, Page 2359*, *13*(14), 2359. https://doi.org/10.3390/ANI13142359

Knight, E., & Przyborski, S. (2015). Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. *Journal of Anatomy*, *227*(6), 746–756. https://doi.org/10.1111/JOA.12257

Komeya, M., Kimura, H., Nakamura, H., Yokonishi, T., Sato, T., Kojima, K., Hayashi, K., Katagiri, K., Yamanaka, H., Sanjo, H., Yao, M., Kamimura, S., Inoue, K., Ogonuki, N., Ogura, A., Fujii, T., & Ogawa, T. (2016). Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Scientific Reports*, *6*. https://doi.org/10.1038/SREP21472

Kubota, H., & Brinster, R. L. (2018). Spermatogonial stem cells. *Biology of Reproduction*, *99*(1), 52–74. https://doi.org/10.1093/BIOLRE/IOY077

Kulibin, A. Y., & Malolina, E. A. (2023). In vitro spermatogenesis: In search of fully defined conditions. *Frontiers in Cell and Developmental Biology*, *11*, 1106111. https://doi.org/10.3389/FCELL.2023.1106111/BIBTEX

Kyriazakis, I., Arndt, C., Aubry, A., Charlier, J., Ezenwa, V. O., Godber, O. F., Krogh, M., Mostert, P. F., Orsel, K., Robinson, M. W., Ryan, F. S., Skuce, P. J., Takahashi, T., Van Middelaar, C. E., Vigors, S., & Morgan, E. R. (2024). Improve animal health to reduce livestock emissions: quantifying an open goal. *Proceedings. Biological Sciences*, *291*(2027). https://doi.org/10.1098/RSPB.2024.0675

Lamb, G. C., & Mercadante, V. R. G. (2016). Synchronization and Artificial Insemination Strategies in Beef Cattle. *The Veterinary Clinics of North America. Food Animal Practice*, *32*(2), 335–347. https://doi.org/10.1016/J.CVFA.2016.01.006

Lengi, A. J., Stewart, J. W., Makris, M., Rhoads, M. L., & Corl, B. A. (2022). Heat Stress Increases Mammary Epithelial Cells and Reduces

Viable Immune Cells in Milk of Dairy Cows. *Animals 2022, Vol. 12, Page 2810*, *12*(20), 2810. https://doi.org/10.3390/ANI12202810

Leslie, S. W., Soon-Sutton, T. L., & Khan, M. A. (2024). Male Infertility. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK562258/

Li, J., Ma, J., Fei, X., Zhang, T., Zhou, J., & Lin, J. (2019). Roles of cell migration and invasion mediated by Twist in endometriosis. *Wiley Online LibraryJ Li, J Ma, X Fei, T Zhang, J Zhou, J LinJournal of Obstetrics and Gynaecology Research, 2019•Wiley Online Library*, *45*(8), 1488–1496. https://doi.org/10.1111/JOG.14001

Li, R., & Albertini, D. F. (2013). The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. *Nature Reviews Molecular Cell Biology*, *14*(3), 141–152. https://doi.org/10.1038/NRM3531;SUBJMETA

Liu, W., Du, L., Li, J., He, Y., & Tang, M. (2024). Microenvironment of spermatogonial stem cells: a key factor in the regulation of spermatogenesis. *Stem Cell Research and Therapy* , *15*(1), 1–22. https://doi.org/10.1186/S13287-024-03893-Z/FIGURES/3

Liu, Z., Wang, K., Nan, X., Yang, L., Wang, Y., Zhang, F., Cai, M., Zhao, Y., & Xiong, B. (2023). Effects of combined addition of 3-nitrooxypropanol and vitamin B12 on methane and propionate production in dairy cows by in vitro-simulated fermentation. *Journal of Dairy Science*, *106*(1), 219–232. https://doi.org/10.3168/JDS.2022-22207

Martin, J. H., Bromfield, E. G., Aitken, R. J., & Nixon, B. (2016). Biochemical alterations in the oocyte in support of early embryonic development. *Cellular and Molecular Life Sciences: CMLS*, *74*(3), 469. https://doi.org/10.1007/S00018-016-2356-1

Matsumura, T., Sato, T., Abe, T., Sanjo, H., Katagiri, K., Kimura, H., Fujii, T., Tanaka, H., Hirabayashi, M., & Ogawa, T. (2021). Rat in vitro spermatogenesis promoted by chemical supplementations and oxygen-tension control. *Scientific Reports*, *11*(1), 1–13. https://doi.org/10.1038/S41598-021-82792-2;SUBJMETA

Menchaca, A., dos Santos-Neto, P. C., Cuadro, F., Souza-Neves, M., & Crispo, M. (2018). From reproductive technologies to genome editing in small ruminants: an embryo's journey. *Animal Reproduction*, *15*(Suppl 1), 984. https://doi.org/10.21451/1984-3143-AR2018-0022

Meuwissen, T. H. E., Hayes, B. J., & Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, *157*(4), 1819–1829. https://doi.org/10.1093/GENETICS/157.4.1819

Mikkola, M., Desmet, K. L. J., Kommisrud, E., & Riegler, M. A. (2024). Recent advancements to increase success in assisted reproductive technologies in cattle. *Animal Reproduction*, *21*(3), e20240031. https://doi.org/10.1590/1984-3143-AR2024-0031

Molinari, P. C. C., Dahl, G. E., Sheldon, I. M., & Bromfield, J. J. (2022). Effect of calving season on metritis incidence and bacterial content of the vagina in dairy cows. *Theriogenology*, *191*, 67–76. https://doi.org/10.1016/J.THERIOGENOLOGY.2022.08.001

Mruk, D. D., & Cheng, C. Y. (2015). The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocrine Reviews*, *36*(5), 564–591. https://doi.org/10.1210/ER.2014-1101

Mueller, M. L., & Van Eenennaam, A. L. (2022). Synergistic power of genomic selection, assisted reproductive technologies, and gene editing to drive genetic improvement of cattle. *CABI Agriculture and Bioscience*, *3*(1), 1–29. https://doi.org/10.1186/S43170-022-00080-Z/FIGURES/5

Nardone, A., Ronchi, B., Lacetera, N., Ranieri, M. S., & Bernabucci, U. (2010). Effects of climate changes on animal production and sustainability of livestock systems. *Livestock Science*, *130*(1–3), 57–69. https://doi.org/10.1016/J.LIVSCI.2010.02.011

Naskar, S., Gowane, G. R., Chopra, A., Paswan, C., & Prince, L. L. L. (2012). Genetic adaptability of livestock to environmental stresses. *Environmental Stress and Amelioration in Livestock Production*,

9783642292057, 317–378. https://doi.org/10.1007/978-3-642-29205-7_13

Nguyen, N. K., Ooi, C. H., Singha, P., Jin, J., Sreejith, K. R., Phan, H. P., & Nguyen, N. T. (2020). Liquid Marbles as Miniature Reactors for Chemical and Biological Applications. *Processes 2020, Vol. 8, Page 793*, *8*(7), 793. https://doi.org/10.3390/PR8070793

Ni, F. Da, Hao, S. L., & Yang, W. X. (2019). Multiple signaling pathways in Sertoli cells: recent findings in spermatogenesis. In *Cell Death and Disease* (Vol. 10, Issue 8). Nature Publishing Group. https://doi.org/10.1038/s41419-019-1782-z

Oatley, J. M. (2018). Recent advances for spermatogonial stem cell transplantation in livestock. *Reproduction, Fertility and Development*, *30*(1), 44–49. https://doi.org/10.1071/RD17418

Özkan, Ş., Vitali, A., Lacetera, N., Amon, B., Bannink, A., Bartley, D. J., Blanco-Penedo, I., de Haas, Y., Dufrasne, I., Elliott, J., Eory, V., Fox, N. J., Garnsworthy, P. C., Gengler, N., Hammami, H., Kyriazakis, I., Leclère, D., Lessire, F., Macleod, M., … Kipling, R. P. (2016). Challenges and priorities for modelling livestock health and pathogens in the context of climate change. *Environmental Research*, *151*, 130–144. https://doi.org/10.1016/J.ENVRES.2016.07.033

Pasquariello, R., Bogliolo, L., Di Filippo, F., Leoni, G. G., Nieddu, S., Podda, A., Brevini, T. A. L., & Gandolfi, F. (2024). Use of assisted reproductive technologies (ARTs) to shorten the generational interval in ruminants: current status and perspectives. *Theriogenology*, *225*, 16–32. https://doi.org/10.1016/j.theriogenology.2024.05.026

Pennarossa, G., Arcuri, S., De Iorio, T., Gandolfi, F., & Brevini, T. A. L. (2021). Current Advances in 3D Tissue and Organ Reconstruction. *International Journal of Molecular Sciences 2021, Vol. 22, Page 830*, *22*(2), 830. https://doi.org/10.3390/IJMS22020830

Pennarossa, G., Arcuri, S., De Iorio, T., Ledda, S., Gandolfi, F., & Brevini, T. A. L. (2023). Combination of epigenetic erasing and mechanical cues to generate human epiBlastoids from adult dermal fibroblasts.

Journal of Assisted Reproduction and Genetics, *40*(5), 1015–1027. https://doi.org/10.1007/S10815-023-02773-4/TABLES/2

Pennarossa, G., Arcuri, S., Zmijewska, A., Orini, E., Gandolfi, F., & Brevini, T. A. L. (2024). Bioengineering-tissue strategies to model mammalian implantation in vitro. *Frontiers in Bioengineering and Biotechnology*, *12*, 1430235. https://doi.org/10.3389/FBIOE.2024.1430235/BIBTEX

Pennarossa, G., Ghiringhelli, M., Gandolfi, F., & Brevini, T. A. L. (2020). Whole-ovary decellularization generates an effective 3D bioscaffold for ovarian bioengineering. *Journal of Assisted Reproduction and Genetics*, *37*(6), 1329–1339. https://doi.org/10.1007/s10815-020-01784-9

Pennarossa, G., Ghiringhelli, M., Gandolfi, F., & Brevini, T. A. L. (2021). Creation of a bioengineered ovary: isolation of female germline stem cells for the repopulation of a decellularized ovarian bioscaffold. *SpringerG Pennarossa, M Ghiringhelli, F Gandolfi, TAL BreviniNext Generation Culture Platforms for Reliable In Vitro Models: Methods and, 2021•Springer*, *2273*, 139–149. https://doi.org/10.1007/978-1-0716-1246-0_9

Pennarossa, G., Ledda, S., Arcuri, S., Gandolfi, F., & Brevini, T. A. L. (2020). A Two-Step Strategy that Combines Epigenetic Modification and Biomechanical Cues to Generate Mammalian Pluripotent Cells. *Journal of Visualized Experiments : JoVE*, *2020*(162). https://doi.org/10.3791/61655

Pennarossa, G., Paffoni, A., Ragni, G., Gandolfi, F., & Brevini, T. A. L. (2020). Rho Signaling-Directed YAP/TAZ Regulation Encourages 3D Spheroid Colony Formation and Boosts Plasticity of Parthenogenetic Stem Cells. *Advances in Experimental Medicine and Biology*, *1237*, 49–60. https://doi.org/10.1007/5584_2019_423

Raj, Y., Mehta, K., & Kumar, R. (2025). *Genetic Selection for Advances in Meat Quality*. https://doi.org/10.5281/Vettoday.14995672

Rasouli, M., Safari, F., Kanani, M. H., & Ahvati, H. (2024). Principles of Hanging Drop Method (Spheroid Formation) in Cell Culture. *Methods in Molecular Biology*, *2879*, 289–300. https://doi.org/10.1007/7651_2024_527

Rauw, W. M., & Gomez-Raya, L. (2015). Genotype by environment interaction and breeding for robustness in livestock. *Frontiers in Genetics*, *6*(OCT), 157050. https://doi.org/10.3389/FGENE.2015.00310/BIBTEX

Reader, K. L., Stanton, J. A. L., & Juengel, J. L. (2017). The Role of Oocyte Organelles in Determining Developmental Competence. *Biology*, *6*(3). https://doi.org/10.3390/BIOLOGY6030035

Rust, J. M. (2018). The impact of climate change on extensive and intensive livestock production systems. *Animal Frontiers: The Review Magazine of Animal Agriculture*, *9*(1), 20. https://doi.org/10.1093/AF/VFY028

Saleem, A., Anwar, S., Nawaz, T., Fahad, S., Saud, S., Ur Rahman, T., Khan, M. N. R., & Nawaz, T. (2025). Securing a sustainable future: the climate change threat to agriculture, food security, and sustainable development goals. *Journal of Umm Al-Qura University for Applied Sciences*, *11*(3), 595–611. https://doi.org/10.1007/S43994-024-00177-3/FIGURES/3

Salonia, A., Bettocchi, C., Boeri, L., Capogrosso, P., Carvalho, J., Cilesiz, N. C., Cocci, A., Corona, G., Dimitropoulos, K., Gül, M., Hatzichristodoulou, G., Jones, T. H., Kadioglu, A., Martínez Salamanca, J. I., Milenkovic, U., Modgil, V., Russo, G. I., Serefoglu, E. C., Tharakan, T., … Minhas, S. (2021). European Association of Urology Guidelines on Sexual and Reproductive Health—2021 Update: Male Sexual Dysfunction. *European Urology*, *80*(3), 333–357. https://doi.org/10.1016/J.EURURO.2021.06.007

Sato, T., Katagiri, K., Kubota, Y., & Ogawa, T. (2013). In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. *Nature Protocols*, *8*(11), 2098–2104. https://doi.org/10.1038/NPROT.2013.138

Sejian, V., Bhatta, R., Gaughan, J. B., Dunshea, F. R., & Lacetera, N. (2018). Review: Adaptation of animals to heat stress. In *Animal* (Vol. 12, Issue s2, pp. S431–S444). Cambridge University Press. https://doi.org/10.1017/S1751731118001945

Shuchat, S., Yossifon, G., & Huleihel, M. (2022). Perfusion in Organ-on-Chip Models and Its Applicability to the Replication of Spermatogenesis In Vitro. *International Journal of Molecular Sciences*, *23*(10), 5402. https://doi.org/10.3390/IJMS23105402

Siu, M. K. Y., & Cheng, C. Y. (2004). Extracellular Matrix: Recent Advances on Its Role in Junction Dynamics in the Seminiferous Epithelium During Spermatogenesis1. *Https://Doi.Org/10.1095/Biolreprod.104.028225*, *71*(2), 375–391. https://doi.org/10.1095/BIOLREPROD.104.028225

Siu, M. K. Y., & Yan Cheng, C. (2009). Extracellular Matrix and Its Role in Spermatogenesis. *Advances in Experimental Medicine and Biology*, *636*, 74–91. https://doi.org/10.1007/978-0-387-09597-4_5

Staub, C., & Johnson, L. (2018). Review: Spermatogenesis in the bull. *Animal*, *12*(s1), s27–s35. https://doi.org/10.1017/S1751731118000435

Staun-Ram, E., & Shalev, E. (2005). Human trophoblast function during the implantation process. *Reproductive Biology and Endocrinology : RB&E*, *3*. https://doi.org/10.1186/1477-7827-3-56

Sultan, M., Zhou, Y., Shamshiri, R. R., Imran, M., & Neethirajan, S. (2023). The Significance and Ethics of Digital Livestock Farming. *AgriEngineering 2023, Vol. 5, Pages 488-505*, *5*(1), 488–505. https://doi.org/10.3390/AGRIENGINEERING5010032

Țogoe, D., & Mincă, N. A. (2024). The Impact of Heat Stress on the Physiological, Productive, and Reproductive Status of Dairy Cows. *Agriculture 2024, Vol. 14, Page 1241*, *14*(8), 1241. https://doi.org/10.3390/AGRICULTURE14081241

Van Eenennaam, A. L. (2019). Application of genome editing in farm animals: cattle. *Transgenic Research*, *28*(2), 93–100. https://doi.org/10.1007/S11248-019-00141-6/FIGURES/8

Van Eenennaam, A. L. (2025). Current and future uses of genetic improvement technologies in livestock breeding programs. *Animal Frontiers : The Review Magazine of Animal Agriculture*, *15*(1), 80–90. https://doi.org/10.1093/AF/VFAE042

Van Eenennaam, A. L., & Young, A. E. (2014). Prevalence and impacts of genetically engineered feedstuffs on livestock populations. *Journal of Animal Science*, *92*(10), 4255–4278. https://doi.org/10.2527/JAS.2014-8124

Velicky, P., Knöfler, M., & Pollheimer, J. (2016). Function and control of human invasive trophoblast subtypes: Intrinsic vs. maternal control. *Cell Adhesion & Migration*, *10*(1–2), 154–162. https://doi.org/10.1080/19336918.2015.1089376

Wadood, A. A., Bordbar, F., & Zhang, X. (2025). Integrating omics approaches in livestock biotechnology: innovations in production and reproductive efficiency. *Frontiers in Animal Science*, *6*, 1551244. https://doi.org/10.3389/FANIM.2025.1551244/FULL

Walke, G., Gaurkar, S. S., Prasad, R., Lohakare, T., & Wanjari, M. (2023). The Impact of Oxidative Stress on Male Reproductive Function: Exploring the Role of Antioxidant Supplementation. *Cureus*, *15*(7), e42583. https://doi.org/10.7759/CUREUS.42583

Wang, P., Suo, L. J., Wang, Y. F., Shang, H., Li, G. X., Hu, J. H., & Li, Q. W. (2014). Effects of GDNF and LIF on mouse spermatogonial stem cells proliferation in vitro. *Cytotechnology*, *66*(2), 309–316. https://doi.org/10.1007/S10616-013-9574-2/METRICS

Wang, X., Li, W., Feng, X., Li, J., Liu, G. E., Fang, L., & Yu, Y. (2023). Harnessing male germline epigenomics for the genetic improvement in cattle. *Journal of Animal Science and Biotechnology*, *14*(1), 1–20. https://doi.org/10.1186/S40104-023-00874-9/TABLES/2

Wankar, A. K., Rindhe, S. N., & Doijad, N. S. (2021). Heat stress in dairy animals and current milk production trends, economics, and future perspectives: the global scenario. *Tropical Animal Health and Production*, *53*(1). https://doi.org/10.1007/S11250-020-02541-X

Washburn, R. L., Hibler, T., Kaur, G., & Dufour, J. M. (2022). Sertoli Cell Immune Regulation: A Double-Edged Sword. *Frontiers in Immunology*, *13*, 913502. https://doi.org/10.3389/FIMMU.2022.913502/FULL

Xiao, Y., Zhang, J., Guan, Y., Wang, M., Liu, D., Xiong, S., Li, J., & Yu, X. (2024). Research progress on Sertoli cell secretion during spermatogenesis. In *Frontiers in Endocrinology* (Vol. 15). Frontiers Media SA. https://doi.org/10.3389/fendo.2024.1456410

Xie, B., Qin, Z., Huang, B., Xie, T., Yao, H., Wei, Y., Yang, X., Shi, D. S., & Jiang, H. (2010). In vitro culture and differentiation of buffalo (Bubalus bubalis) Spermatogonia. *Reproduction in Domestic Animals*, *45*(2), 275–282. https://doi.org/10.1111/j.1439-0531.2008.01281.x

Xu, H. Q., Liu, J. C., Zhang, Z. Y., & Xu, C. X. (2022). A review on cell damage, viability, and functionality during 3D bioprinting. *Military Medical Research*, *9*(1), 1–15. https://doi.org/10.1186/S40779-022-00429-5/TABLES/1

Yang, L., Di, A., Song, L., Liu, X., Wu, D., Wang, S., Hao, Z., Bu, L., Bai, C., Su, G., Wei, Z., Zhang, L., Liu, Z., Gao, S., & Li, G. (2025). Generation of modified cows and sheep from spermatid-like haploid embryonic stem cells. *Nature Biotechnology*, 1–9. https://doi.org/10.1038/S41587-025-02832-4;SUBJMETA

Yoshida, S. (2008). Spermatogenic stem cell system in the mouse testis. *Cold Spring Harbor Symposia on Quantitative Biology*, *73*, 25–32. https://doi.org/10.1101/SQB.2008.73.046

Yoshida, S. (2020). Mouse Spermatogenesis Reflects the Unity and Diversity of Tissue Stem Cell Niche Systems. *Cold Spring Harbor*

Perspectives in Biology, *12*(12), 1–20. https://doi.org/10.1101/CSHPERSPECT.A036186

Yoshida, S., Sukeno, M., & Nabeshima, Y. I. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science (New York, N.Y.)*, *317*(5845), 1722–1726. https://doi.org/10.1126/SCIENCE.1144885