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Dissociation, enrichment, and the *in vitro* formation of gonocyte colonies from cryopreserved neonatal bovine testicular tissues

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ABSTRACT

Gonocytes play an important role in early development of spermatogonial stem cells and fertility preservation to acquire more high quality gonocytes *in vitro* for further germ cell-related research and applications, it is necessarily needed to enrich and *in vitro* propagate gonocytes from cryopreserved bovine testicular tissues. This study aimed to investigate the isolation, enrichment, and colony formation of gonocytes *in vitro* for germ cell expansion from cryopreserved neonatal bovine testicular tissues. The effects of several different *in vitro* culture conditions, including seeding density, temperature, serum replacement and extracellular matrices were investigated for the maintenance, proliferation and formation of gonocyte colonies *in vitro*. Frozen/thawed two-week-old neonatal bovine testicular tissues were digested and gonocytes were enriched using a Percoll density gradient. Cell viability was accessed by trypan blue staining and cell apoptosis was evaluated by TUNEL assays. Gonocytes were identified and confirmed by immunofluorescence with the PGP9.5 germ cell marker and the OCT4 pluripotency marker while Sertoli cells were stained with vimentin. We found that neonatal bovine gonocytes were efficiently enriched by a 30%–40% Percoll density gradient ($p < 0.05$). No significant differences were detected between neonatal bovine testicular cells cultured at 34 °C or 37 °C. The formation of gonocyte colonies was observed in culture medium supplemented with knockout serum replacement (KSR), but not fetal bovine serum (FBS), at a seeding density higher than 5.0×10^4 cells/well. A greater number of gonocyte colonies were observed in culture plates coated with laminin (38.00 ± 6.24/well) and Matrigel (38.67 ± 3.78/well) when compared to plates coated with collagen IV and fibronectin ($p < 0.05$). In conclusion, bovine neonatal gonocytes were able to be efficiently isolated, enriched and maintained in gonocyte colonies *in vitro*; the development of this protocol provides vital information for the clinical translation of this technology and the future restoration of human fertility.

1. Introduction

Primordial germ cells (PGCs), gonocytes/prospermatogonia (hereafter referred to as gonocytes) and spermatogonial stem cells (SSCs) all exhibit stem cell potential and are unipotent solely for the generation of sperm; consequently, these cells are referred to as male germline stem cells (mGSCs) [1]. Derived from primordial germ cells (PGCs), gonocytes are the primitive germ cells that exist in the early stages of the neonatal testis after birth and give rise to SSCs [2]. Gonocytes are formed during the fetal period and remain largely quiescent until the early postnatal period. After birth, gonocytes gradually migrate from the center to the basement membrane of the seminiferous cords and resume proliferation, subsequently developing into SSCs. The development of PGCs to gonocytes and the transition from gonocytes to SSCs are important because the dysfunctional development of germ cells often leads to infertility [3]. SSCs are fundamental for spermatogenesis as they possess the capability of self-renewal to sustain SSC pools and undergo differentiation into spermatozoa [4].

In this manuscript, we focused on the *in vitro* culture of gonocytes from frozen/thawed neonatal bovine testicular tissues. It is critical that we perform such work in animal models before we can translate our protocols to the preservation of pre-pubertal human testicular tissue to protect and rescue the fertility of pubertal males with cancer. The bovine model serves as a highly appropriate system for our investigations

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because of their comparable testicular morphological characteristics and testicular microenvironment. In Holstein bulls, the initiation of spermatogenesis begins at approximately 20 weeks and completes by 32 weeks [5], thus allowing us to select the appropriate age to represent puberty.

The genetic makeup and reproductive output of both cattle and humans is heavily reliant on the quality and quantity of gonocytes and SSCs in the testicular microenvironment. However, only a limited number of studies have attempted to preserve bovine gonocytes or propagate these cells *in vitro* [6,7]. To enhance or apply techniques related to germ cells, it is necessary to isolate a substantial quantity of germ cells. Consequently, there is an urgent need to develop methods for the *in vitro* enrichment and propagation of gonocytes/SSCs in the bovine model to serve as a foundation for future translation to clinical research and the preservation of fertility in prepubertal males with cancer. The specific investigation of gonocytes has important implications for fertility preservation and restoration in clinical patients, basic reproductive research in animals, as well as stem cell technology and therapy.

The acquisition of fresh immature testicular tissues (ITTs) from large domestic animals, such as cattle, for research purposes is challenging and costly when compared to small rodents which do not represent suitable models for humans. Therefore, there is a crucial need to develop methods to cryopreserve ITTs and expand populations of gonocytes and SSCs to facilitate the application of germ cell-related techniques and the development of fertility preservation protocols in cattle [7], endangered animals, and eventually, humans. Moreover, research has shown that abnormality or failure of the transition from gonocytes to SSCs may lead to testicular germ cell tumors [8,9]. However, the underlying mechanisms behind this phenomenon remain largely unknown. Thus, investigating the mechanisms and regulatory factors associated with gonocyte development is fundamental for germ cell and cancer research. The absence of an effective *in vitro* culture system for preserving and expanding gonocytes/SSCs from frozen/thawed ITTs poses significant challenges with respect to the investigation of key molecular mechanisms underlying the functionality of gonocytes and their transformation into spermatogonia.

With regards to prepubertal cancer patients, the cryopreservation of ITTs is crucial if we are to preserve their future reproductive function [10]; research programmes, such as those developed at the University of Oxford are at the forefront of this exciting area of clinical research. The Oxford Cell and Tissue Biobank (OCTB) has been employing a computer-programmed testicular tissue cryopreservation program to preserve human ITTs since 2013 [10]. This initiative plays a vital role in ensuring the preservation of fertility for individuals undergoing cancer treatments [11] and provides an appropriate foundation from which to develop research protocols to generate viable sperm from thawed ITTs as an option to rescue the fertility of prepubertal males with cancer who undergo gonadotoxic treatments before they have begun to produce viable sperm.

In the present study, we froze neonatal bovine testicular tissues using an established computer-controlled testicular tissue cryopreservation programme at the OCTB. By performing this research on frozen/thawed bovine ITTs, we aimed to (1) optimize the isolation and enrichment of gonocytes from frozen/thawed neonatal bovine testicular tissues, and (2) evaluate various *in vitro* culture conditions for the maintenance and proliferation of gonocytes as well as the formation of gonocyte colonies for the future restoration of fertility.

2. Methods and materials

2.1. Collection and preparation of bovine ITTs

Freshly excised testes were obtained from two-week-old Holstein calves (Bos taurus) from the Tockenham Corner Abattoir (Swindon, UK). Testes were washed three times with transport medium consisting of sterile Hank's balanced salt solution (HBSS; Sigma-Aldrich, UK) supplemented with 2% penicillin-streptomycin (Pen-Strep; Gibco, USA), 0.1 M sucrose (Sigma-Aldrich, UK) and 10% fetal bovine serum (BSA; Sigma-Aldrich, UK). Testes were then immersed in transport medium and transferred immediately on ice to our laboratory. After the removal of adipose tissue and the tunica albuginea, longitudinal incisions were made on each testis to expose the testicular parenchyma. ITTs were then dissected into fragments and then subjected to speed-controlled freezing.

2.2. Freezing and thawing

To freeze testicular tissue fragments, each piece of testicular tissue ($3 \times 3 \times 3$ mm^3 in size) was submerged in a cryovial filled with 1 ml of cryoprotectant medium consisting of transport medium and 1.5 M dimethyl sulfoxide (DMSO; Sigma-Aldrich, UK). Cryovials then underwent a standardized testicular tissue program in a programmable freezer (Sy-lab IceCube, Purkersdorf, Austria) in the Oxford Cell & Tissue Bank (OCTB) [11]. When the program was completed, cryovials were removed from the chamber and rapidly placed into a liquid nitrogen (LN_2)-containing Dewar flask for storage until further analysis. When thawing, the slow-frozen vials were removed from LN_2 and immersed in a 37 °C water bath for 2 min. Then, 1 ml of warm transport medium was immediately added to each vial to dilute the cryomedium by 50%. All contents were then transferred from cryovials to a prepared sterile dish for 3 min. Next, small pieces of testicular tissues were transferred to a fresh dish containing 3 ml of transport medium for 5 min incubation with gentle agitation at 37 °C. This process was repeated twice.

2.3. Isolation of testicular cells

The isolation of testicular cells was optimized based on a previously published two-step enzymatic digestion method [12]. In brief, frozen/-thawed ITT fragments were mechanically desegregated by a disposable scalpel and needles in 2 ml of the first enzymatic solution [0.1% w/v collagenase I-A (Sigma-Aldrich, UK) and 1% (vol/vol) Pen–Strep (Gibco) in minimum essential medium (MEM)-α (Thermo Fisher, UK)] followed by incubation at 37 °C with gentle shaking. After settling for 10 min, the supernatant was collected and washed with MEM-α. Then, the sediment was collected and further digested in 2 ml of a second enzymatic solution [0.1% w/v collagenase I-A (Sigma-Aldrich, UK), 0.05% w/v DNAse I (Sigma-Aldrich, UK), 0.05% w/v hyaluronidase (Sigma-Aldrich, UK) and 1% (vol/vol) Pen–Strep in MEM-α] at 37 °C with gentle shaking. Dissociated cells were then washed and resuspended in fresh MEM-α containing 1% (v/v) Pen–Strep.

2.4. Enrichment of gonocytes

After enzymatic digestion, dissociated testicular cells were suspended in a 5% Percoll density gradient (Hunter Scientific, UK) which was constructed by layering 2 ml of different percentages (50%, 45%, 40%, 35%, 30% and 20%) of Percoll in a 15 ml Falcon tube. The cell layer was carefully placed onto the Percoll density gradient followed by centrifugation for 25 min at 1000 g and 37 °C. Cells within and between different Percoll density phases were collected, washed with 4–5 vol of phosphate-buffered saline (PBS) and thoroughly mixed by inversion before centrifugation at 700*g* for 5 min at 37 °C. After removal of the supernatant, cells in the pellets were ready for further processing.

Differential plating was used to determine the most effective incubation time and coating method for gonocyte enrichment by seeding freshly isolated testicular cells on plates either coated or uncoated with 2% Matrigel at a density of 1×10^5 cell/well. Testicular cells were fed with 10% knockout serum replacement (KSR)-minimum essential medium (MEM)-α and cultured at 37 °C with 5% CO_2 for 3 h or overnight. Non-adherent cells suspended in the culture medium were collected by brief centrifugation at 1200 rpm at room temperature for 5 min. Adherent cells were then observed under a microscope. The proportions

of gonocytes were then determined by immunocytochemical staining with PGP9.5.

2.5. Cell culture

Gonocytes collected by different enrichment methods were analyzed for cell viability using trypan blue staining before being seeded for further experiments. Four extracellular matrixes (ECM), including laminin (Corning, UK), collagen IV (Corning, UK), Matrigel (Corning, UK) and fibronectin (Corning, UK), were diluted in PBS separately to generate 5 μg/cm^2 coating solutions which were then used to coat plates for 1 h at 37 °C. Enriched gonocytes were cultured in 24-well culture dishes coated with one of the four ECM using either MEM-α or Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% KSR or 10% fetal bovine serum (FBS). Culture medium was changed every 2 days.

2.6. Immunofluorescence

Immunofluorescence staining was performed using an anti-PGP9.5 (also known as UCHL-1) antibody (ab72911, Abcam, UK) to validate the germ cell status of gonocytes [13,14], an anti-OCT4 (also known as POU5F1) antibody (NB100-2379, Novus biological, UK) to evaluate the pluripotency of gonocytes [15–17], and anti-Ki67 antibody (ab15580, Abcam, UK) to identify proliferating cells. Anti-vimentin antibody (sc-6260; Santa Cruz Biotechnology, Germany) was used to identify Sertoli cells. In brief, the coverslips/slides with resuspended cell smears or adhered cells/colonies were fixed by 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). Samples were then permeabilized with 0.1% Triton X-100 (v/v) (Sigma-Aldrich, UK) for 15 min and then blocked using 1% BSA in PBS for 1 h at RT. Samples were then incubated with primary antibodies (1:100 for PGP9.5 and OCT4; 1:200 for Ki67 and vimentin) at 4 °C overnight followed by washing and incubation with secondary antibody goat anti-mouse/rabbit IgG (1:200; Abcam, UK) for 1 h at RT. All samples were mounted using Vectashield antifade-mounting medium with DAPI (Vector Lab, UK) prior to fluorescence microscopic evaluation.

2.7. Cell viability and apoptosis

Cell viability was assayed with trypan blue staining using a hemocytometer. A fluorometric terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (DeadEnd; Promega, Fitchburg, WI, USA) was used to detect and quantify apoptotic cells by measuring nuclear DNA fragmentation in accordance with the manufacturer's instructions. Samples were analyzed under a fluorescence microscope (Nikon, Tokyo, Japan) and ten random fields from each slide were imaged. Imaging data were analyzed by ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

2.8. Statistical analysis

The Shapiro–Wilk test was used to test the normality of quantitative data. Data with a normal distribution are presented as a mean ± standard deviation (SD), whereas data with a non-normal distribution are presented as a median (25% percentile, 75% percentile). GraphPad Prism 9.3.0 software (GraphPad Software, San Diego, California, USA) was used to create graphs and perform tests for statistical significance. Statistical differences between groups were analyzed by a Student's *t*-test or analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Isolation, enrichment and the identification of gonocytes and Sertoli cells

Firstly, a two-step enzymatic method was optimized by evaluating the incubation time by comparing 20 and 30 min for both the first and second enzymatic solutions. Analysis showed that incubation for 30 min for each step on a shaker effectively isolated testicular fragments into single cells while maintaining a high cell viability of 85.00 ± 4.00%; incomplete tissue digestion occurred after incubation for 20 min (Fig. 1A). After three days of culture in a dish, gonocytes showed a typical germ cell appearance with a larger cell size, a typical round shape and a higher nucleus/cytoplasm ratio (Fig. 1B). Gonocytes were characterized by immunocytochemical staining using the PGP9.5 germ cell marker and the OCT4 pluripotency stem cell marker (Fig. 1C). Somatic cells formed a confluent feeder monolayer to support gonocyte growth at the surface. Sertoli cells were detected by vimentin staining (Fig. 1D).

Next, isolated cells were subjected to discontinuous Percoll density gradient centrifugation to compare the effectiveness of 20%, 30%, 35%, 40%, 45% and 50% Percoll density gradients in gonocyte enrichment. The proportion of PGP9.5-positive cells was used to determine the effectiveness of gonocyte enrichment. The 30–45% Percoll fractions contained significantly higher proportions of PGP9.5-positive cells compared to the other fractions with values of 49.8% ± 1.833 in the 30% fraction, 42.36% ± 2.17 in the 35% fraction, 32.127% ± 3.4 in the 40% fraction, and 23.1% ± 1.49 in the 45% fraction compared to that in the total testicular cells (7.87 ± 2.215%) ($p < 0.05$) (Fig. 2A). There was no significant difference in viability when compared between testicular cells isolated from different fractions of the Percoll gradient ($p > 0.05$) (Fig. 2B). Therefore, 30–40% Percoll was used for subsequent germ cell enrichments due to its efficacy for selecting germ cells. The proportion of PGP9.5-positive cells was 45.10% ± 3.67 after 30–40% Percoll gradient enrichment compared to 7.87% ± 2.215 in the total testicular cells ($p < 0.001$). Percoll gradient (30–40%) centrifugation led to a significantly lower proportion (41.37% ± 2.62) of vimentin-positive cells ($p < 0.05$) compared to that in the total testicular cells prior to enrichment (81.72% ± 3.48) (Fig. 2C). There were no significant differences in viability or apoptosis in testicular cells with or without 30–40% Percoll selection ($p > 0.05$) (Fig. 2D).

Differential plating was also used for gonocyte enrichment. Dissociated testicular cells were cultured for 3 h or overnight on uncoated or Matrigel-coated plates prior to the collection of suspended cells. The bottom of each culture dish was examined by brightfield microscopy after the removal of suspended cells. After 3 h of primary cell culture, the bottom of Matrigel-coated or uncoated plates showed little cell attachment (Fig. 3A). Increased levels of somatic cell attachment were observed on Matrigel-coated or uncoated plates subjected to overnight primary cell culture. Both round germ cells and flat somatic cells were attached to the bottom of plates after the removal of suspended cells following overnight incubation with or without Matrigel coating on differential plates. The proportion of gonocytes (PGP9.5-positive) was 14.00% ± 1.23 among non-adherent cells after overnight incubation on uncoated differential plates and 18.10% ± 1.02 among adherent cells after overnight incubation on Matrigel-coated differential plates; both these proportions were significantly higher than that for total testicular cells (8.33% ± 1.53; $p < 0.05$; Fig. 3B).

3.2. Effects of cell seeding density on the formation of colonies

To determine the optimal seeding density for culturing neonatal gonocytes *in vitro*, comparisons were made by seeding enriched testicular cells into 24-well plates at densities of 1.0×10^4 cells/well, 5.0×10^4 cells/well or 1.0×10^5 cells/well. Cells were cultured at 34 °C and the culture medium (MEM-α + 10% KSR) was changed every 2 days.

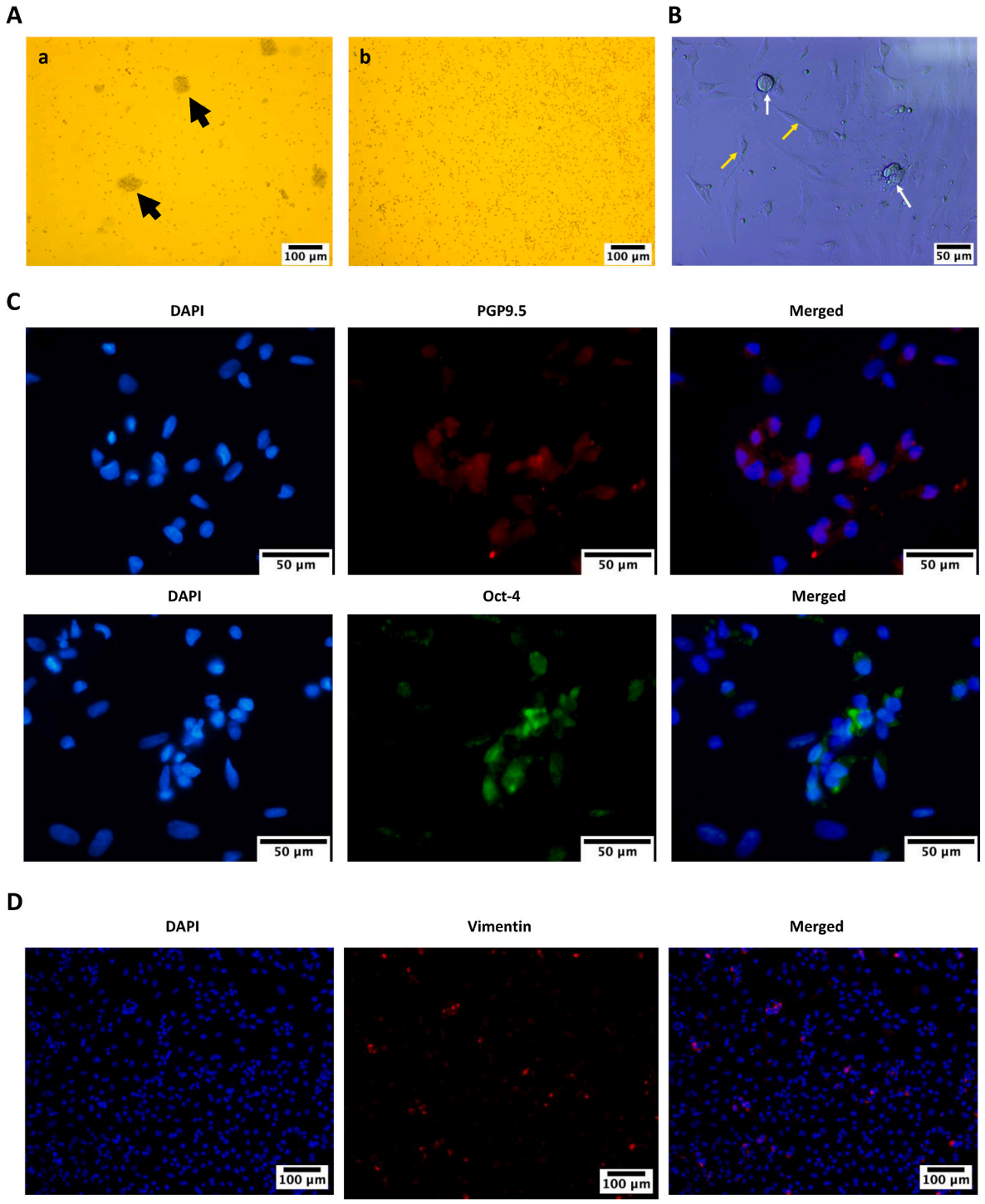

Fig. 1. Dissociation of testicular cells from neonatal bovine testicular tissues and the characterization of bovine gonocytes. (A) Cells isolated from cryopreserved neonatal bovine testicular samples using two-step enzymatic digestion. Small clumps of cells were observed with a 20 min incubation time in the first and second steps of the digestion process (white arrow). Single isolated cells were observed with a 30 min incubation time for both steps of the digestion process. Scale bar = 100 μm. (B) Brightfield image of enriched gonocytes/SSCs. White arrows indicate gonocytes/SSCs, and yellow arrows indicate Sertoli somatic cells. (C) Representative immunofluorescence images of enriched gonocytes/SSCs revealing the expression of PGP9.5 and OCT4. Scale bar = 50 μm. (D) Representative immunofluorescence images of vimentin positive Sertoli cells in culture. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Cells with a seeding density of 1.0×10^5 cells/well became confluent significantly earlier than those with 1.0×10^4 cells/well ($p < 0.05$), reaching 90% confluence in 8.83 ± 0.75 days (Table 1). The formation of colonies was observed in cells seeded at higher density but not in cells seeded at 1.0×10^4 cells/well. Gonocyte colonies initially appeared at 5.31 ± 0.64 days in the 1.0×10^5 cells/well group and 7.42 ± 1.45 days in the 5.0×10^4 cells/well group. In subsequent experiments, a seeding density of 1.0×10^5 cells/well was used due to the shorter time required to reach confluency and the appearance of colony formation.

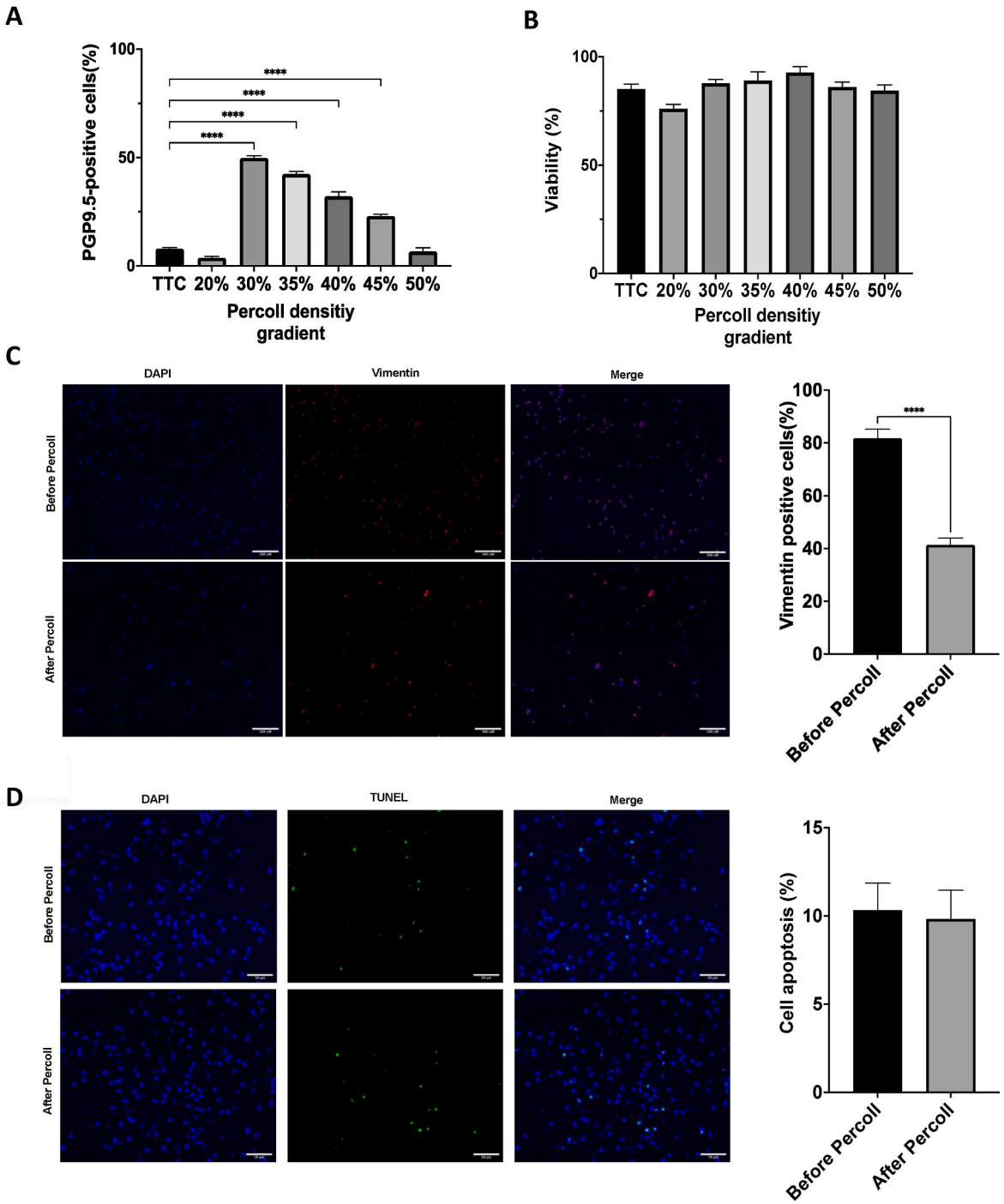

Fig. 2. Purification of bovine gonocytes. (A) Proportion of PGP9.5-positive cells in testicular cells selected by 20%, 30%, 35%, 40%, 45% and 50% Percoll density gradients. TTC, total testicular cells. (B) Viability of cells selected by different Percoll density gradients. (C) Proportion (%) of vimentin-positive cells in testicular cells before and after 30%–40% Percoll gradient selection. Scale bar = 100 μM. (D) Cell apoptosis as assessed by TUNEL assay in testicular cells before and after 30%–40% Percoll gradient selection. Scale bar = 50 μm. Three biological replicates were performed (n = 3). Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

3.3. Effects of culture medium and serum-free culture on the formation of colonies

The effects of different culture media (DMEM/F12 and MEM-α) supplemented with 10% FBS or 10% KSR were evaluated for gonocyte culture. Cells were seeded in 24-well plates and maintained for 10 days without passage. There were no significant differences for the time taken to reach confluency days when compared across the four groups (Table 2). The formation of "grape/dome-shaped" colonies was only observed in the DMEM/F12 + 10% KSR and MEM-α + 10% KSR groups (Fig. 4A); these colonies expressed the *PGP9.5* SSC marker and the OCT4 (PGC marker) as confirmed by immunofluorescence (Fig. 4B). Gonocyte colonies initially appeared at 7.15 ± 1.09 days in the DMEM/F12 + 10% KSR group; this was significantly later than the 5.33 ± 0.70 days for the MEM-α + 10% KSR group ($p < 0.05$). The numbers and diameters of gonocyte colonies were compared on day 7 because seeded cells started to become confluent on day 4 after culture. The mean number of gonocyte colonies was 7.42 ± 0.81 colonies/well in the MEM-α + 10%

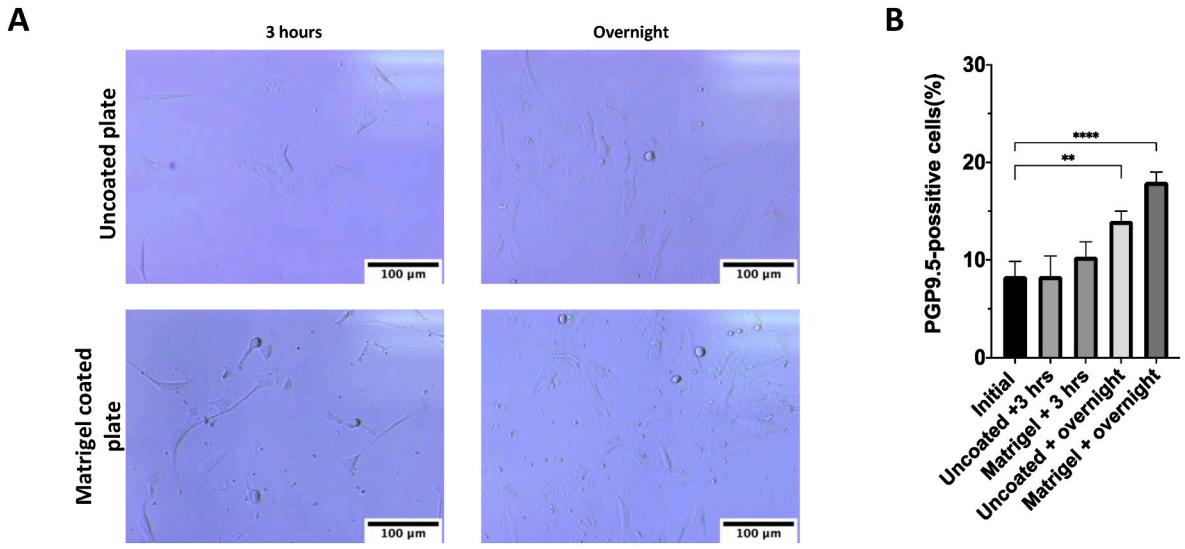

Fig. 3. Enrichment of bovine gonocytes by differential plating. (A) Representative brightfield images of adherent cells after incubation for 3 h or overnight incubation on uncoated or Matrigel-coated differential plates. (B) Proportions of PGP9.5-positive cells in the initial group (total testicular cells) and groups of non-adherent cells on uncoated differential plates with a 3 h incubation (uncoated + 3 h), Matrigel-coated plates with a 3 h incubation (Matrigel + 3 h), uncoated plates with overnight incubation (uncoated ± overnight), and Matrigel-coated plates with overnight incubation (Matrigel + overnight). Three biological replicates were performed (n = 3). Data are presented as the mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Table 1
Effects of three cell seeding densities on the growth of testicular cells in culture.

	1.0×10^4 cells/well	5.0×10^4 cells/well	1.0×10^5 cells/well
Day confluency was reached	13.67 ± 1.37*	11.33 ± 0.86	8.83 ± 0.75*
Day colonies initially appeared	–	7.42 ± 1.45	5.31 ± 0.64
Viability (%)	90.33 ± 2.16	90.50 ± 2.42	92.50 ± 2.28

The results are shown as mean ± SD. One-way analysis of variance (ANOVA) was used for statistical analysis; multiple comparisons were performed for different columns within rows. *Significantly different ($p < 0.05$) within day confluency was reached and between columns.

Table 2
Effects of different culture media with and without serum on the formation of bovine gonocyte colonies.

	DMEM/F12 + 10% FBS	DMEM/F12 + 10% KSR	MEM-α + 10% FBS	MEM-α + 10% KSR
Day confluency was reached	8.72 ± 1.16	8.96 ± 0.71	8.65 ± 1.23	9.20 ± 0.80
Day colonies initially appeared	n/a	7.15 ± 1.09[a]	n/a	5.33 ± 0.70[a]
Number of colonies per well at Day 7	n/a	2.75 ± 0.54[b]	n/a	7.42 ± 0.81[b]
Diameter of colonies at Day 7 (μm)	n/a	60.53 ± 10.72	n/a	63.76 ± 11.27

MEM-α, Minimum Essential Medium-alpha; DMEM/F12, Dulbecco's modified Eagle's medium nutrient mixture F-12; FBS, fetal bovine serum; KSR, knockout serum replacement. Data are presented as the mean ± SD. Data labelled [a] or [b] were compared and significant differences identified ($p < 0.05$).

KSR group; this was significantly higher than the 2.75 ± 0.54 colonies/well in the DMEM/F12 + 10% KSR group ($p < 0.05$). However, there was no significant difference in the mean colony diameter when compared between the two groups. Therefore, MEM-α + 10% KSR was selected for use in subsequent experiments.

3.4. Comparison of two incubation temperatures for the formation of colonies

Next, we evaluated the effects of two different incubation temperatures (34 °C *vs* 37 °C) on the growth of cultured testicular cells. There were no significant differences in the day at which cells reached confluency, the day at which colonies initially appeared, the numbers of colonies, the diameters of colonies or the viability of cells when compared between the two incubation temperature groups (Table 3). Because the mean spermatid cord temperature is approximately 34 °C in bulls [18], 34 °C was selected for subsequent experiments.

3.5. Effects of different ECM components on short-term in vitro gonocyte culture

The main components of the basement membrane in seminiferous tubules are laminin, collagen IV and fibronectin. Therefore, we evaluated the effects of these four types of ECM on the culture of bovine gonocytes by evaluating the morphological changes of enriched bovine testicular cells after 7 days of culture (Fig. 5A). By 24 h after cell seeding, suspended cells began to adhere to the bottom of the culture dish; these adhered cells were mostly somatic cells. In the next few days, dome-shaped, refractile colonies started to form; these were surrounded by flat somatic cells. Somatic cells in the collagen IV and fibronectin groups were flatter than those in the Matrigel and laminin groups. During culture, the adherence of colonies to the bottom of the culture dish was looser in the laminin group when compared to the other three groups.

In the laminin group, the number of gonocyte colonies per culture dish on day 3 was 44.00 ± 2.00, whereas only a few colonies appeared in the other groups by this time with 8.67 ± 0.57, 9.00 ± 1.73 and 7.00 ± 2.00 colonies per culture dish in the collagen IV, Matrigel and fibronectin groups, respectively ($p < 0.05$) (Fig. 5B). The mean diameter of gonocyte colonies was 78.65 ± 19.71 μm in the laminin group on day 3; this was significantly higher than that in the other three groups ($p < 0.05$). On Day 5, there were significantly more gonocyte colonies in the laminin group (40.67 ± 4.04/well) and Matrigel group (45.33 ± 4.16/well) when compared to the other groups ($p < 0.05$). However, there were no differences among the four groups in terms of colony diameter. On day 7, the number of gonocyte colonies was 38.67 ± 3.78/well in the Matrigel group and 38.00 ± 6.24/well in the laminin group; these

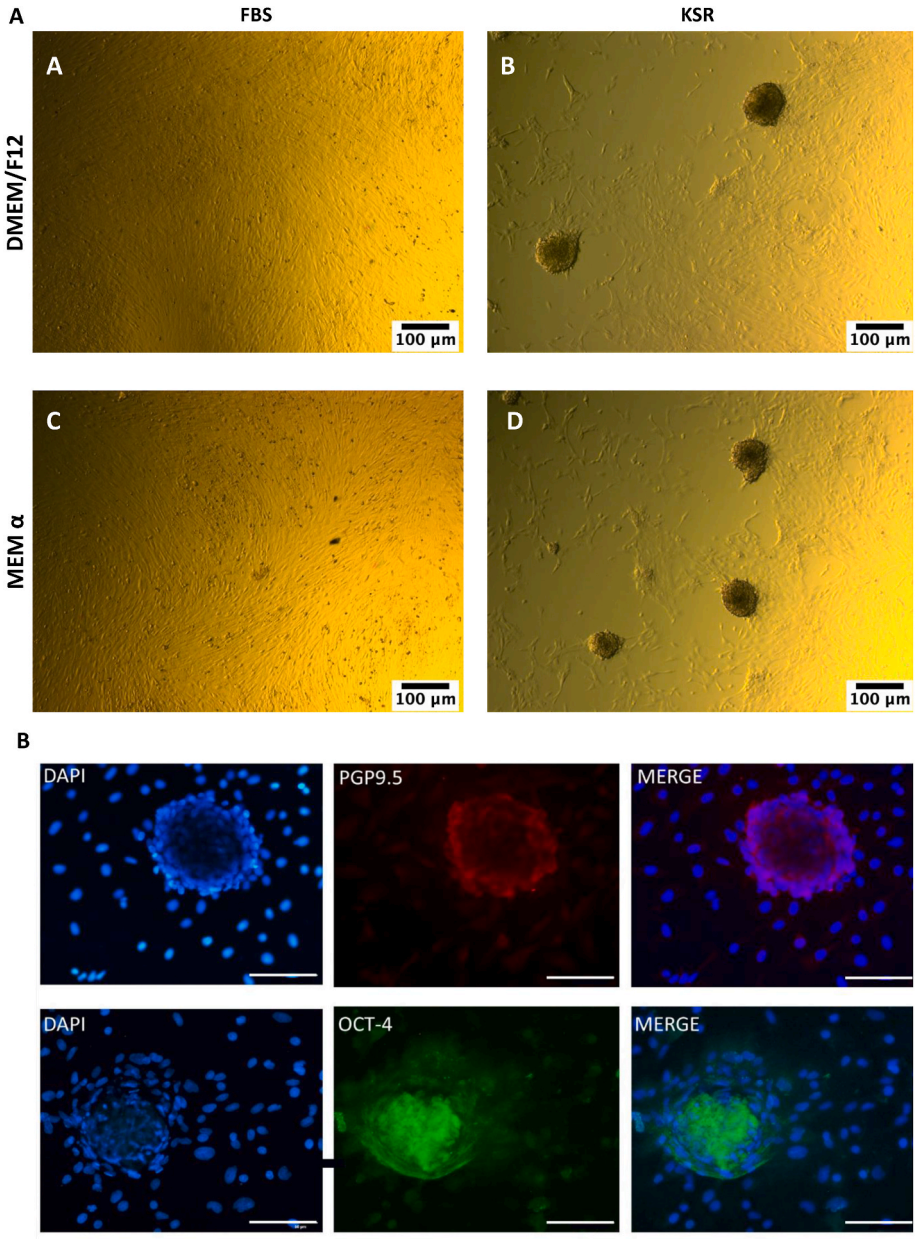

Fig. 4. Formation and characterization of germ cell colonies. (A) Representative images of enriched gonocyte culture in DMEM/F12 or MEM-α culture medium supplemented with knockout serum replacement (KSR) or fetal bovine serum (FBS). (B) Representative immunofluorescence analysis of gonocyte colonies. Positive staining for the PGP9.5 SSC marker. Positive staining for the OCT4 stem cell marker. Scale bar = 100 μm.

Table 3
Effects of two incubation temperatures on the growth and viability of testicular cells in short-term culture.

	34 °C	37 °C
Day confluency was reached	9.12 ± 0.83	9.13 ± 0.79
Day colonies initially appeared	5.24 ± 1.10	5.95 ± 0.93
Number of colonies per well at Day 7	7.15 ± 0.94	7.71 ± 1.02
Diameter of colonies at Day 7	67.43 ± 11.32	62.01 ± 12.12
Viability (%)	92.31 ± 2.86	90.93 ± 3.30

Data are presented as the mean ± SD. *$p < 0.05$.

values were significantly higher than the collagen IV (10.33 ± 1.52/well) and fibronectin (7.67 ± 1.58/well) groups ($p < 0.05$). The mean diameter of colonies in the collagen group on day 7 was 162.3 ± 64.0 μm; this was significantly higher than the colony diameters in the other groups ($p < 0.05$) (Fig. 5C). The diameters of colonies in the Matrigel and laminin groups increased at a steady rate with a smaller variation in colony size.

TUNEL-positive cells were found both at the edges of the gonocyte colonies and among uncolonized somatic cells. There were significant increases in the proportion of TUNEL-positive cells on day 7 of culture compared to day 1 of culture in all four ECM groups ($p < 0.05$); however, there were no differences among the four groups on day 7 (Fig. 5D).

4. Discussion

Cryopreservation, encompassing both speed-controlled and non-speed-controlled methods, is widely applied to preserve fertility and conduct research on both animals and humans. Previous studies have utilized an insulated container, with non-speed-controlled cooling rate, to freeze testicular tissues from neonatal bulls [7,19]. In this study, we developed a pioneering protocol that utilized a clinically established

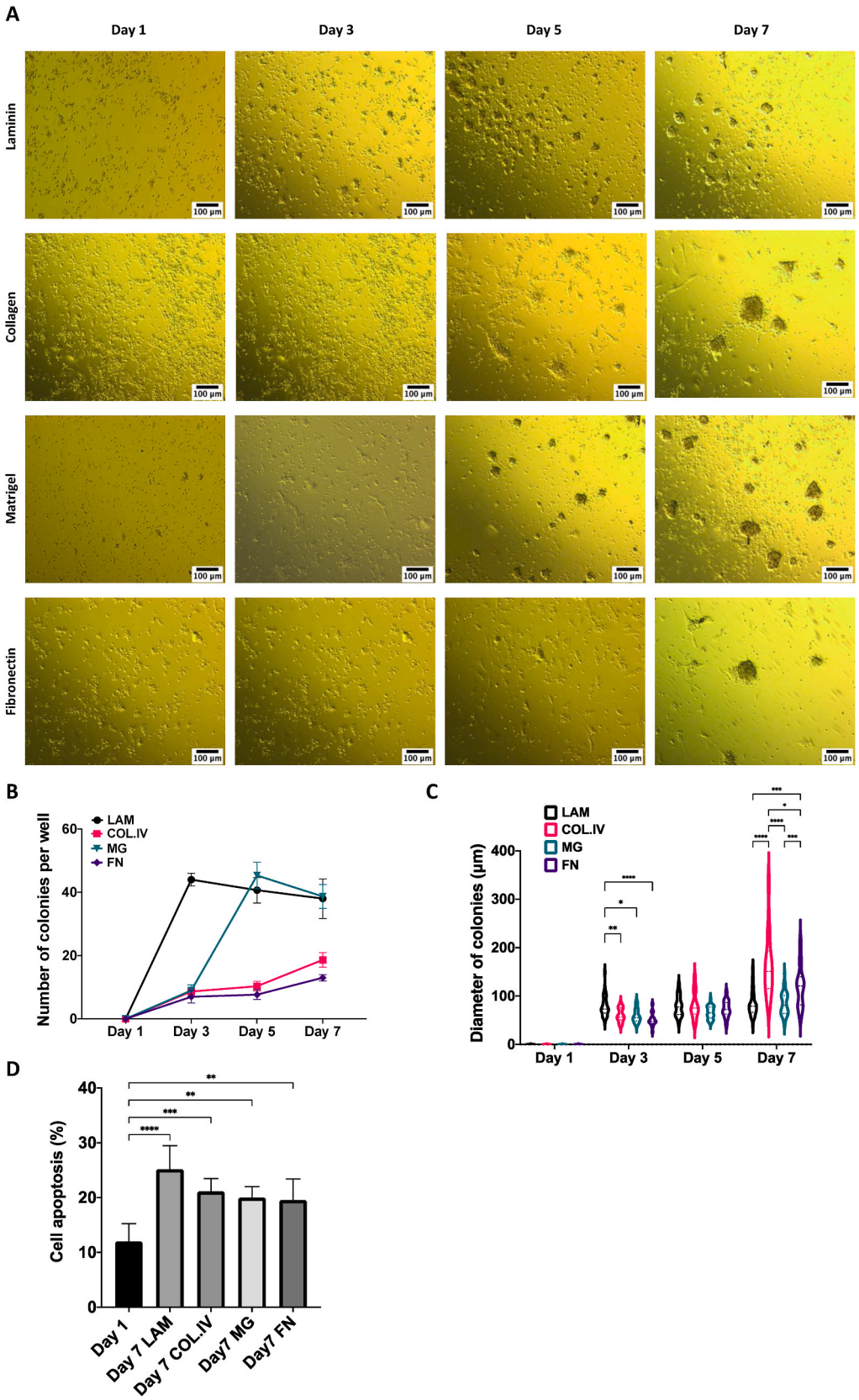

Fig. 5. Growth of gonocyte/SSC colonies on four extracellular matrices. (A) Representative brightfield images of enriched gonocyte culture on laminin (LAM), collagen IV (COL.IV), Matrigel (MG), and fibronectin (FN) from Day 1 to Day 7 of culture. (B) Numbers and (C) diameters of colonies formed per well on the four extracellular matrices. Scale bar = 100 μm $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

speed-controlled and computer-based cryopreservation program at the OCTB to freeze testicular tissues from neonatal bulls. The implementation of this speed-controlled program ensures a more stable, consistent, and controllable cooling rate for neonatal bovine testicular tissues, thus providing a more consistent effect when compared to uncontrolled-speed freezing protocols. Our findings provide compelling evidence that this protocol effectively preserves the high viability of neonatal bovine testicular cells, thus indicating the potential application of speed-controlled freezing protocols for bovine gonocytes. Furthermore, our findings provide a key foundation for the future translation of our new protocol to the preservation of testicular tissue from prepubertal males undergoing cancer treatment. This strategy provides significant options for the future restoration of fertility in prepubertal males with cancer.

The development of efficient techniques for germ cell isolation and enrichment could potentially enable germ cell transplantation or *in vitro* spermatogenesis. A two-step enzymatic method has been widely used to dissociate neonatal [2], prepubertal [20,21], peripubertal [22] and adult bovine testicular tissues, as well as testicular tissues from sheep [23], mice [24] and humans [25]. In the present study, we modified a two-step enzymatic tissue dissociation protocol and demonstrated that a 30 min incubation time was optimal for testicular tissues acquired from two-week-old neonatal bulls while maintaining high levels of cell viability.

Several methods have been used for the enrichment of undifferentiated bovine SSCs. For example, discontinuous Percoll density centrifugation selection has been used to isolate undifferentiated SSCs or gonocytes from prepubertal or adult bulls. However, different proportions of Percoll density gradients were used for the enrichment of undifferentiated spermatogonial stem cells in other studies, including 20% [26], 20% and 40% [2], and 35%–45% [27]. Here, our results showed that 30–40% Percoll gradient density centrifugation significantly increased the proportion of gonocytes from neonatal testicular tissues without significant changes in cell viability or apoptosis. For differential plating, laminin has been proposed for SSC selection due to the presence of α-6 integrin receptors on the surface of mouse and bovine SSCs [2,28,29]. Germ cells take longer to adhere to the bottom of plates and therefore can be enriched in non-adherent cells [30]. Gonocytes do not easily adhere or bind to ECM proteins because they are located in the center of the seminiferous cords; therefore, negative selection was used in the present study to isolate gonocytes from somatic cells. The present results indicated that differential plating enriched bovine gonocytes and reduced the proportion of Sertoli cells. In addition, increases in the proportion of gonocytes were observed among suspended cells after overnight incubation in both Matrigel-coated and uncoated differential plates. However, the suitable adherence time may vary and should be optimized; otherwise, a certain number of the gonocytes adhered to plates may be lost [31]. Morphology-based selection is a simple and low-cost method for the isolation of gonocytes. Because somatic cells grow faster than SSCs in culture, it is necessary to check for and mechanically isolate SSC colonies for further passage and culture before the SSCs are overgrown by other cell types, thus requiring a highly efficient technique [32]. In some cases, colonies of gonocytes cannot be easily visualized or are mixed with somatic cells; this can lead to the loss of gonocytes. Overall, discontinuous Percoll gradient density selection is regarded as a controllable, simple and time-saving procedure for gonocyte enrichment.

Primary testicular cells are sensitive to certain materials that are present in the serum, thus suggesting that serum-free culture medium is a beneficial option for primary cell culture [33]. For *in vitro* spermatogenesis, KSR has been used in a culture medium to successfully induce the maturation of rat germ cells to the round spermatid stage [34]. Lipid-rich BSA (AlbuMAX) has been suggested as the most critical component in KSR for *in vitro* spermatogenesis in mice [35] and for the *in vitro* self-renewal of bovine gonocytes [27]. In the present study, our results also supported the fact that KSR is a key factor in the formation of "grape/dome-shaped" bovine gonocyte colonies during *in vitro* 2D culture; this is consistent with previous results arising from the culture of mouse SSCs [33] and the expansion of bovine gonocytes [27]. A previous study reported the presence of bovine germ cells, confirmed by RT-PCR, in "distinct embryonic stem (ES) cell-like" colonies for up to 13 passages when cultured with a medium supplemented with KSR [27]. Similarly, Gholami, Pourmand [36] reported that the culture of human germ cell colonies in soft agar with KSR and hormones enhanced the number and size of colonies as well as promoting the differentiation of germ cells. In addition, KSR supplemented with melatonin and GlutaMAX has been successfully used for *in vitro* spermatogenesis (IVS) in mice with an organ culture method [37]. Therefore, KSR is recommended as a serum replacement for the *in vitro* propagation of bovine gonocytes. However, the regulatory mechanisms underlying the signaling pathways involved in the KSR-mediated formation and propagation of gonocyte colonies remains largely unknown and requires further investigation.

In vivo, spermatogenesis occurs inside the testes at a temperature that is 2–7 °C lower than body temperature. High temperatures suppress the self-renewal of SSCs but not cellular apoptosis [38]. An incubation temperature of 37 °C has been reported to lead to an increased loss of tubular morphology and intratubular cell death in human testicular tissue culture on days 14 and 70 [39]. In the present study, we compared culture temperatures of 34 °C and 37 °C with respect to the enrichment of neonatal bovine gonocytes; no significant differences were found between these two temperatures in terms of cell viability and cell proliferation rate. This lack of temperature effects may be attributed to the fact that we used neonatal (2-week-old) bovine testes; at this time, shortly after birth, the fetal calf testis is ready to descend, thus indicating that the testicular cells are not sensitive to temperature.

In mammals, the epithelial cells in the testicular tubules exhibit an intimate relationship with ECM proteins [40]. The components of the basement membrane of the seminiferous tubules include laminin, collagen IV and fibronectin [41]. The ECM provides essential physical support for cells, thus allowing for cell-cell communications and cell migration. Therefore, it is important to provide necessary ECM components for the *in vitro* culture of testicular cells to support appropriate organization and interaction. In the present study, laminin, collagen IV, Matrigel and fibronectin were compared with respect to the optimal culture and colony formation of gonocytes from frozen/thawed testicular tissues. Enriched cells cultured on laminin- or Matrigel-coated plates formed colonies at a faster rate; this may be associated with interactions between the integrin cell surface receptor and laminin. Exposure to laminin can change the morphology of testicular cells and affect the migration of cells during culture by upregulating the expression of molecules involved in cell migration, the secretion of cytokines, the induction of hypoxia response and PI3K/Akt/mTOR signaling [42]. Overall, enriched gonocytes formed higher numbers of moderately sized colonies on laminin- and Matrigel-coated plates compared to collagen IV- and fibronectin-coated plates. Because Matrigel better maintained gonocyte colonies on plates for use in subsequent procedures, such as staining or collection, Matrigel was recommended for the *in vitro* culture of neonatal bovine gonocytes based on our findings. Previous studies compared the culture of gonocytes from fresh testicular tissues obtained from neonate [43] and 3-month-old Holstein bulls [44]. Li et al. [43] showed that the rate of colony formation was higher in Laminin than Matrigel; our present results support this finding but only within the first 3 days of culture; on the fifth day, the numbers of colonies were similar when compared between Laminin and Matrigel. In another study [44], gonocyte culture was conducted on plates precoated with Dolichos biflorus agglutinin (DAB), gelatin, laminin, and poly-L-lysine. This previous study demonstrated that the number of germ cell colonies on plates pre-coated with DAB and poly-L-lysine was higher than on the other two coatings. However, the mechanisms underlying morphological and phenotypic changes in bovine gonocytes, when influenced by the ECM and other niche growth factors involved in self-renewal or

differentiation, remain largely unknown and require further investigation.

There are some limitations of this study that need to be considered. For example, we mainly optimized the culture conditions for short-term maintenance of gonocytes *in vitro*. However, *in vitro* spermatogenesis from gonocytes to sperm and the potential maturation of other somatic cells were not investigated; further investigations need to address this issue.

5. Conclusion

In the present study, we developed an optimized two-step digestion and 30–40% Percoll density gradient centrifugation strategy to efficiently isolate and enrich neonatal bovine gonocytes. A basic medium consisting of DMEM/F12 or MEM-α + 10% KSR and certain ECM components (mainly laminin and Matrigel) significantly enhanced the formation of bovine gonocyte colonies. Importantly, under optimized conditions, both cultured gonocytes and gonocyte colonies expressed specific germ cell markers and pluripotency markers. Overall, we optimized the basic culture conditions that can be used to increase germ cell numbers and expand their colonies *in vitro*, thereby shedding light on further downstream germ cell-related applications for fertility preservation and regenerative medicine.

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CRediT authorship contribution statement

Shiyan Tang: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, preparation. **Celine Jones:** Supervision, Conceptualization, Methodology, Resources, Writing – review & editing. **Julian Dye:** Conceptualization. **Kevin Coward:** Supervision, Conceptualization, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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