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Generation of healthy bovine ovarian organoids: a proof-of-concept derivation technique

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Abstract

Background Organoids have emerged as powerful tools in reproductive medicine and bioengineering, offering three-dimensional (3D) models that closely mimic native tissues. However, the development of protocols for generating healthy epithelial ovarian organoids (OvaOs) remains significantly underexplored, as most studies focus on ovarian cancer models. This work presents an effective protocol for generating healthy bovine OvaOs as a physiological and translational model for ovarian research, mimicking the anatomical and functional similarities between bovine and human ovarian surface epithelium (OSE).

Results Healthy bovine OvaOs were successfully derived using a mechanical-enzymatic method with a predominant mechanical approach, which proved superior to exclusively enzymatic techniques that failed to yield an adequate number of OSE cells. The biological potential of the resulting OvaOs to establish long-term organoid lines was demonstrated by their exponential growth over a 21-day culture period, extensive passaging capacity, and high viability after freeze-thaw cycles. Histological analyses confirmed that healthy bovine OvaOs recapitulated OSE tissue characteristics, including the expression of Cytokeratin 18, Vimentin, and CD44, while the absence of Paired box gene-8 (PAX8) expression excluded contamination by fimbrial cells.

Conclusions This study describes an effective mechanical protocol for deriving healthy OvaOs from bovine ovaries. These 3D models faithfully replicate the biological features of bovine OSE, with sustained viability across long-term cultures, passaging, and freeze-thaw cycles. These findings underscore their potential as translational models for advancing ovarian physiology research and adapting protocols to human ovarian tissue.

Keywords Bovine ovary, Ovarian surface epithelium, Healthy ovarian organoids, 3D ovarian organoid culture

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Introduction

Organoids represent a groundbreaking tool in reproductive biology, enabling the development of three-dimensional (3D) in vitro models that closely recapitulate the structure and function of native tissues [1]. These models are typically generated from stem or progenitor cells embedded in a scaffold —either natural, synthetic or hybrid— that mimics the tissular microenvironment by providing essential mechanical and structural support, together with biochemical signals. In addition, organoid cultures require specialized culture media enriched with growth factors to support cell proliferation and spatial organization [2]. In contrast to conventional 2D in vitro cultures, organoids more faithfully replicate in vivo conditions by preserving tissue-specific architecture and complex cellular interactions, making them an invaluable resource for understanding the biological features of the native organ [3].

In particular, epithelial organoids from the female reproductive tract —such as the endometrium and fallopian tubes— have been successfully established [4–6], providing significant insights into both physiological and pathological processes [7–10]. However, despite these advances, the development of protocols for generating healthy epithelial ovarian organoids (OvaOs) remains significantly underexplored.

In the literature, most of current approaches focus on OvaOs derived from cancer tissues, primarily of epithelial origin, which severely limits their application for modelling normal ovarian physiology [11–13]. These cancer studies often require the generation of non-malignant OvaOs as experimental controls; however, specific methodological details regarding their derivation are rarely provided. Furthermore, the direct application of OvaOs protocols from cancer biopsies to healthy ovarian samples is challenging due to the significantly lower abundance of epithelial cells in normal ovaries compared to epithelial ovarian carcinomas. This discrepancy need for tailored methodologies to effectively isolate, expand, and culture healthy OvaOs from healthy tissues. Although several studies have described the development of healthy OvaOs from both mice [14] and human [15] ovaries, these protocols require the immediate digestion of fresh intact ovaries. As a result, they are not compatible with cryopreserved ovarian cortex samples, which significantly complicates processing logistics. Furthermore, the inherent complexity of the ovary —comprising a dynamic interaction between epithelial, stromal, and germ cells— difficult the isolation and 3D culture of ovarian epithelial cells in a specific and reproducible manner [16].

The ovarian surface epithelium (OSE) plays a key structural role in the organ and is actively involved in crucial biological processes such as ovulation and repair mechanisms following follicular rupture [17]. Due to their embryonic origin, OSE cells express both epithelial markers, like cytokeratin 18 (CK18); and mesenchymal markers, such as vimentin (VIM), reflecting their dual nature. Other specific marker of this cell population is CD44, a cell adhesion molecule that plays crucial roles in cell-cell and cell-matrix interactions, tissue repair, and cellular processes like proliferation and survival [18]. In contrast to fimbrial cells from the end of the oviduct, OSE do not express the Paired box gene-8 protein (PAX8) [19]. These unique characteristics, observed in humans and similarly in other large mammals, are essential for the successful development of healthy and pathologic OvaOs models.

In this context, the anatomical and physiological similarities between bovine and human ovaries [20, 21] — including morphology, size, follicular dynamics, and hormonal signaling — make the bovine ovary an ideal tissue source for establishing robust OvaOs derivation protocols, which can later be adapted to human ovarian bioengineering. In particular, the OSE from both species consists of a single layer of cuboidal epithelial cells that play structural and mechanical functions, including the maintenance of ovarian integrity and facilitation of follicular rupture during ovulation [16]. Despite its proximity to the ovarian stroma, the OSE demonstrates a relatively limited responsiveness to endocrine signals, suggesting a predominantly mechanosensitive rather than hormone-driven regulation [21]. Furthermore, OSE cells in both species are embedded within a structurally conserved extracellular matrix (ECM), primarily composed of collagen and hyaluronic acid, alongside other glycoproteins and proteoglycans that contribute to cellular adhesion, migration, and tissue remodeling [22, 23].

This proof-of-concept study describes, for the first time, an effective technique for generating healthy OvaOs lines from bovine ovaries. These organoids can be successfully long-term cultured, passaged, frozen, and thawed with high cell viability. Like other organoid models, they faithfully mimic their native tissue by expressing key epithelial, mesenchymal, and stemness-associated markers, reproducing the characteristics of bovine OSE tissue.

Results

The mechanical-enzymatic protocol for obtaining bovine OSE cells and generating bovine OvaOs

At day 0, mechanical scraping of bovine ovaries produced over 10^6 viable cells/mL, sufficient to seed 8–10 Matrigel droplets in a 48-well plate. In addition to this mechanical step, a light enzymatic digestion was performed to ensure cell individualization. In contrast, the exclusively enzymatic method produced a markedly lower cell yield, probably due to the challenges associated with digesting

the highly rigid ovarian cortex (Fig. 1A and C). By day 4 of culture, these cells began organizing into spherical structures, forming bovine OvaOs (Fig. 1B and D). Notably, the mechanical protocol resulted in a significantly higher number of bovine OvaOs (80 ± 5) compared to the exclusively enzymatic method (10 ± 3), which was ultimately discarded due to its insufficient productivity (Fig. 1). Given the substantial differences in terms of efficiency between both protocols, the bovine OvaOs generated using the mechanical-enzymatic method were selected for all subsequent studies, including passaging, freezing-thaw, and OvaOs characterization (Fig. 1C-K).

To confirm the epithelial origin of cells, an immunocytochemical analysis for CK18 was performed after mechanical scraping. The CK18 positive expression was not only confirmed in bovine ovarian cells on day 0

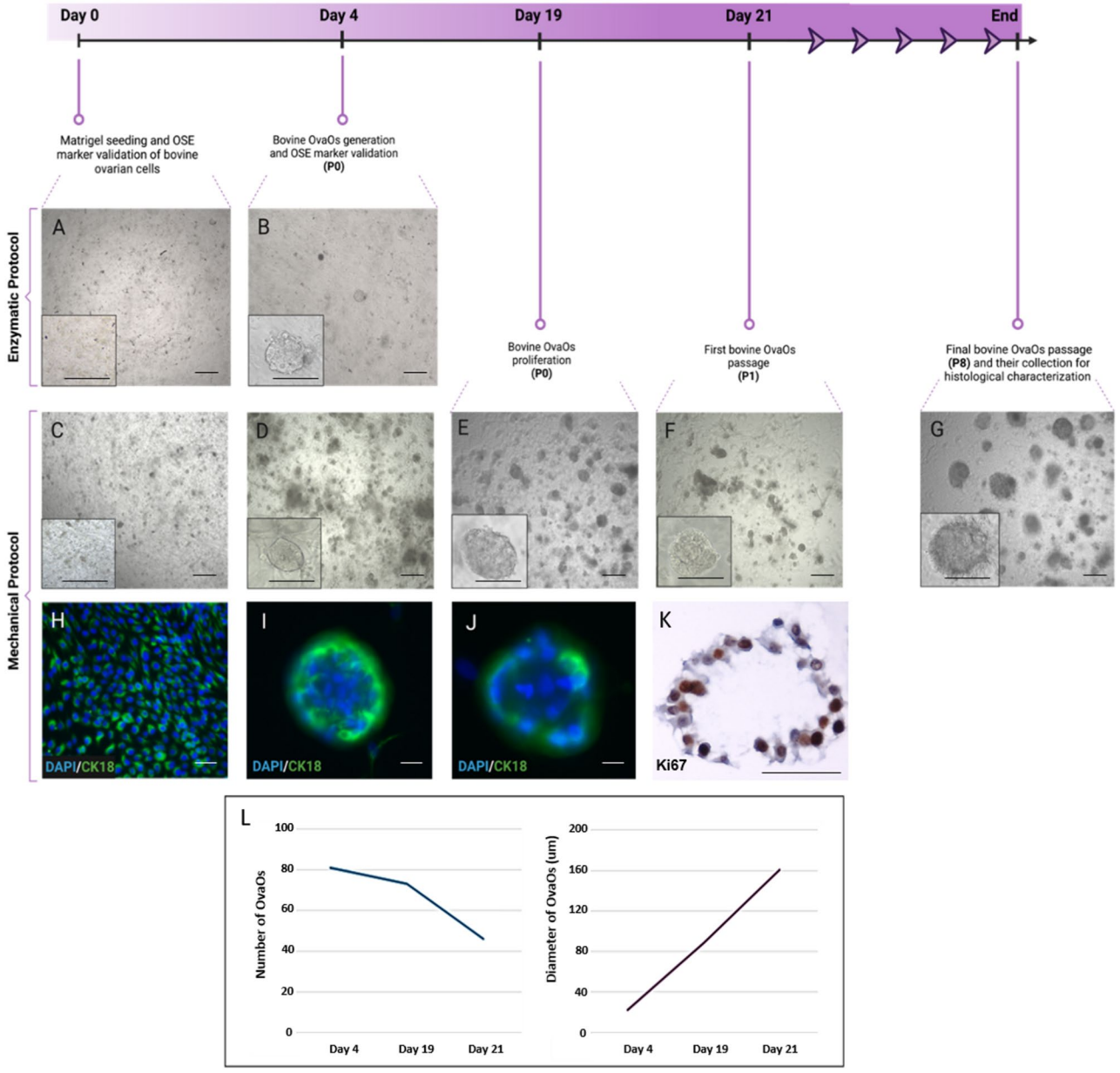


Fig. 1 Experimental timeline for bovine OvaOs generation and characterization. The timeline outlines the key steps: initial culture (Day 0), organoid formation and proliferation (Days 4-19), and passaging (Day 21 to end of the experiment). **(A, B)** The enzymatic protocol was discarded due to low cell yield and organoid formation effectiveness. **(C-K)** The mechanical method led to significant bovine OvaOs generation, where the bovine ovarian cells obtained, once seeded **(C)**, were CK18 positive **(H)**, confirming their epithelial origin, similar to the organoids that grew from them **(I, J)**. Organoids were evaluated by bright-field microscopy **(A-G)**, immunocytochemistry for CK18 **(H-J)**, and immunohistochemistry for Ki67 proliferation marker **(K)**, showing an increase in size over time in culture **(K)**. Scale bar (A-G) = 200 µm; scale bar (H-J) = 50 µm; scale bar (zoom images) = 50 µm. *DAPI: 4',6-diamidino-2-phenylindole; CK18: cytokeratin 18; OvaOs: epithelial ovarian organoids; P0: passage 0; P1: passage 1; P8: passage 8*

(Fig. 1H), but also after OvaOs formation and proliferation (Fig 1I and J). These results proved that bovine OSE cells were successfully isolated from the ovarian cortex and finally formed the organoid structures after Matrigel seeding.

Establishment of bovine OvaOs lines: long-term culture, passaging and freezing-thawing capabilities with high cell viability

The potential of bovine OvaOs as established cell lines was evaluated by long-term culture, freezing-thaw, and passaging efficiency. To note, bovine OvaOs exhibited sustained viability over a 21-day culture period. Between Day 0 and Day 4, a notable increase in the number of bovine OvaOs was observed, followed by a slight reduction toward the end of the culture period. This reduction can be attributed to the concurrent increase in the organoid size, which expanded from an average diameter of 22 µm (Day 4) to 160 µm (Day 21) (Fig. 1L). Interestingly, they maintained their high proliferation capacity after passaging, which was confirmed by Ki67 expression marker (Fig. 1K).

On Day 21, two distinct procedures were performed: some bovines OvaOs underwent freezing-thaw (Fig. 2A and D), while others were subjected to culture passaging (Fig. 2B and E). Both methods demonstrated high cell viability, with freezing-thaw survival rates of 92% and post-passaging rates of 97% (Fig. 2G). Notably, bovine OvaOs were successfully passaged every 21 days, up to passage 8 (Fig. 2C and F), while maintaining high survival rate (94%) (Fig. 2G). These findings underline the essential properties required for the establishment of stable organoid lines, highlighting the viability and adaptability of bovine OvaOs for future applications in ovarian bioengineering and research.

Histological characterization of bovine OvaOs: how similar are they to the native tissue?

The histological analysis of the bovine OSE and bovine OvaOs demonstrated remarkable structural and tissular similarities. First, H&E staining revealed the typical columnar epithelium of the native OSE and the well-organized, spherical architecture of the OvaOs, highlighting their comparable cell morphology (Fig. 3A and B). Immunofluorescence analysis confirmed the co-expression of epithelial (CK18, green) and mesenchymal (VIM, red) markers in both the OSE and OvaOs (Fig. 3C and D), reflecting the dual nature of these cells and validating the preservation of their native characteristics within the organoid models. Similarly, CD44 expression, indicative of cell adhesion properties, was detected in both the native OSE and OvaOs (Fig. 3E and F), further emphasizing their phenotypic consistency. In contrast, PAX8 was not expressed in either the bovine OSE or the derived OvaOs (Fig. 3G and H), as expected given its specificity to fimbrial epithelium. This marker was exclusively detected at the cyst level of the bovine ovarian cortex. Positive and negative controls are included in the Supplementary Material section (Fig. 5, Supplementary Material). All these findings validate the histological characteristics of the healthy OvaOs and their close resemblance to the native bovine OSE, which are summarized in Fig. 3.

Methods

Ethical approval declarations Not applicable.

Experimental design

Bovine ovaries were processed using both exclusively enzymatic and mechanical-enzymatic dissociation protocols. However, bovine healthy OvaOs were only successfully generated through the mechanical-enzymatic method. To assess their potential as long-term organoid lines, eight serial passages were conducted, along with freezing and thawing cycles, both evaluated by cell viability assays. Histological characterization of the OvaOs was performed using hematoxylin and eosin (H&E) staining, while immunofluorescence (IF) and immunohistochemistry (IHC) were employed to assess the expression of key OSE markers, including CK18, VIM, and CD44. To discuss the specificity of the technique and detect the presence of fimbrial cells of the oviduct, the PAX8 marker was also tested (Fig. 4).

Bovine ovary processing and OvaOs culture: enzymatic and mechanical-enzymatic protocols

Ovaries from cows (11–14 months old) were donated by a slaughterhouse in Terneras Eliseo S.L (Valencia, Spain) and transported in a pre-warmed washing solution (Table 1). Two distinct protocols were established for ovarian tissue processing: an exclusively enzymatic method and a mechanical-enzymatic protocol.

The enzymatic protocol was performed by following the protocol from Maenhoudt et al. 2021 with slight modifications [11]. Fragments of the ovarian cortex from a pool of ovaries ($n = 5$) were isolated, washed in PBS, and incubated in a collagenase IV solution (Gibco, 17104-019, 1 mg/ml) at 37 °C for 1–2 h in agitation. After enzymatic digestion, tissue suspension was filtered through a 70-µm cell strainer to isolate the OSE population. Finally, OSE were seeded (30,000–50,000 cells/well) in Matrigel (Corning™, 354234) as previously described and cultured in 250 µl of OvaOsCM supplemented with RI (10 µM) (Sigma Aldrich, 688001). Culture media were changed every 3 days.

For the mechanical-enzymatic protocol, a pool of ovaries ($n = 5$) was first washed in PBS 1X to remove excess blood. Then, ovaries were transferred to a Petri dish

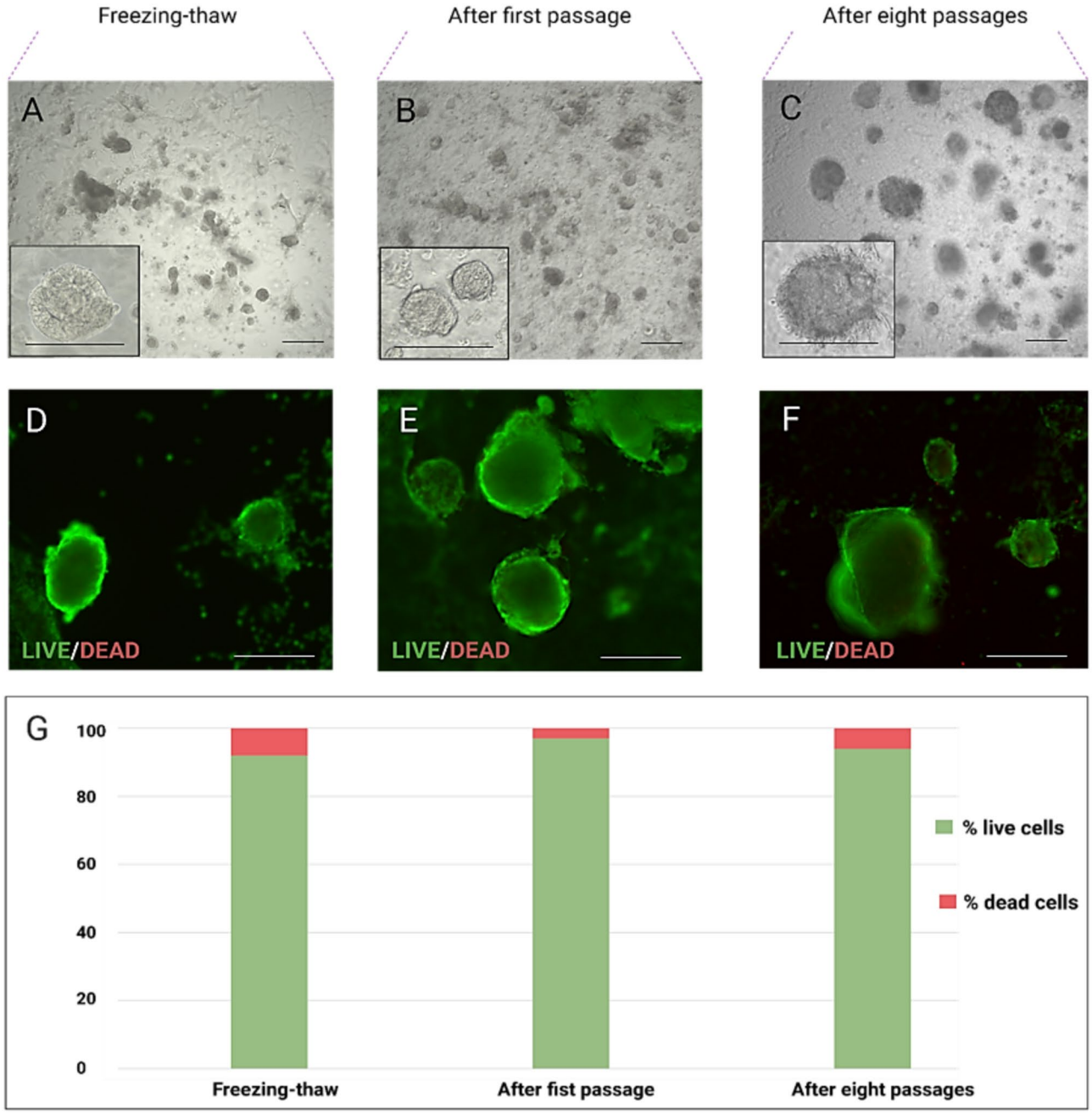
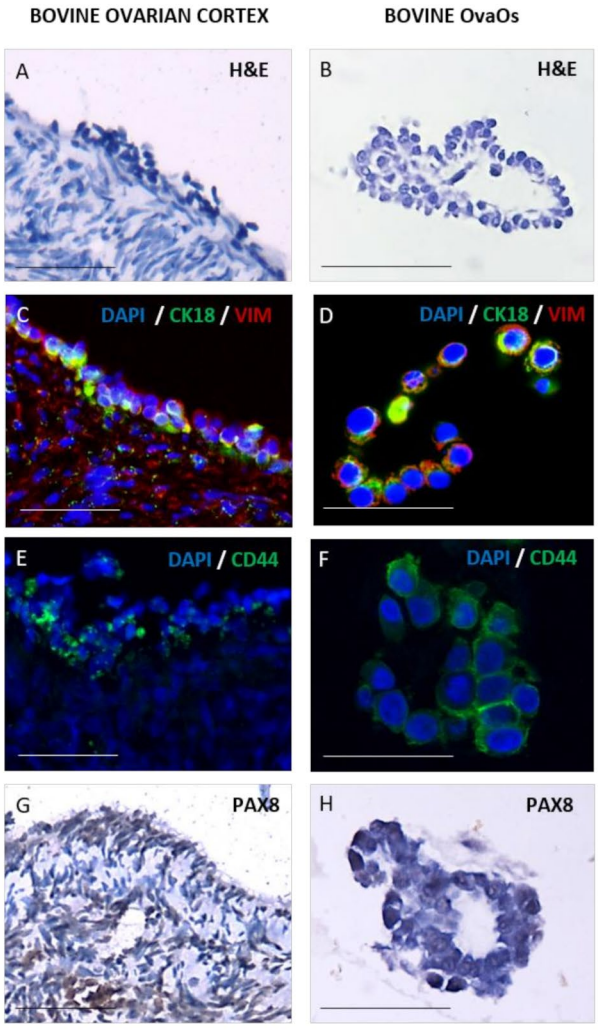


Fig. 2 Evaluation of bovine OvaOs viability after freezing-thaw and passaging. **(A**, **B**, **C)** Bright field images of bovine OvaOs during different experimental conditions: post freezing-thaw **(A)**, post passaging **(B)**, and after eight culture passages **(C)**. **(D**, **E**, **F)** LIVE/DEAD staining of bovine OvaOs corresponding to each condition, with viable cells stained green and dead cells stained red. **(G)** Quantification of cell viability under the different conditions, demonstrating high survival rates post-freezing (92%), post-passaging (97%), and after eight passages (94%). Scale bar = 200 µm; scale bar (zoom images) = 50 µm

with basic medium (Table 1), and the epithelial layer was scraped with a scalpel to release OSE cells. The cell-laden medium was collected and centrifuged (1500 rpm, 5 min). After discarding the supernatant, lysis buffer was used to lysate erythrocytes. After this step, cell solution was filtered with a 70-µm cell strainer (Corning, 352350) to isolate OSE. If OSE aggregations remains intact, a brief collagenase IV digestion step (30 min) is recommended to facilitate their breakdown into single cells. Before seeding, OSE were mixed with DMEM F12 (15%) and combined with Matrigel (85%) [11]. The final mixture was plated in 20 µl/drop in a 48-well plate (Fisher Scientific, 11820765) and allowed to gel at 37 °C for 20 min. Finally, 250 µl of ovarian organoids' culture medium (OvaOsCM) (Table 1) supplemented with RI (10 µm) was added to each well. The composition of OvaOsCM was



MARKER	OVARIAN CORTEX	OVARIAN ORGANOID
CK18	+	+
VIM	+	+
CD44	+	+
PAX8	-	-

Fig. 3 Histological characterization of bovine OSE and derived OvaOs. **(A, B)** H&E staining reveals the characteristic columnar structure of the native bovine OSE **(A)** and the spherical organization of the OvaOs **(B)**. **(C, D)** Immunofluorescence staining (merge) shows co-expression of CK18 (green) and VIM (red) in the bovine OSE **(C)** and bovine OvaOs **(D)**. **(E, F)** CD44 expression (green) is also observed in both the native bovine OSE **(E)** and bovine OvaOs **(F)**. **(G, H)** PAX8 staining is absent in both the bovine OSE **(G)** and bovine OvaOs **(H)**, consistent with the epithelial origin of the OSE cells. All markers expressed in bovine OvaOs and bovine ovarian cortex are summarized in the table. Scale bar = 50 µm. *DAPI: 4′,6-diamidino-2-phenylindole; CK18: cytokeratin 18; VIM: vimentin; PAX8: Paired box gene-8; H&E: Hematoxylin and eosin staining*

selected based on prior comparisons of different formulations from previous studies [11, 14, 15]. Culture media were changed every 3 days.

Long-term culture, passaging, cryopreservation, and thawing of bovine OvaOs

All these procedures were performed by following established protocols with slight modifications [11]. Passaging was performed when organoids reached a 75–80% confluence to avoid the disruption of Matrigel drops. First, the culture medium of each well was removed. Then, 200 µl of cold DMEM F12 was added. The bottom of the wells was scraped to disrupt Matrigel drops, which were then collected and centrifuged (2200 rpm, 5 min). After centrifugation, the supernatant was removed, and 500 µl of TrypLE Select Express (Fisher Scientific, 11528856) was added, followed by incubation for 6–7 min at 37 °C. Subsequently, an equal volume of DMEM F12 was added, and the tubes were centrifuged (2200 rpm, 5 min). The supernatant was removed, 200 µl of DMEM F12 was added, and 100 pipetting actions were performed to mechanically disrupt the organoids. Finally, the organoids were mixed with DMEM F12 (15%), seeded in Matrigel (85%) and cultured in OvaOsCM. Culture media were refreshed every 3 days. Organoids were passaged at confluence and Matrigel disruption, approximately every 20 days. Notably, bovine OvaOs were passaged up to eight times without any negative impact on their formation and proliferation capacity.

For the cryopreservation process, ovarian organoids were collected, centrifuged, and enzymatically disrupted as similar protocols previously described [11]. Then, the pellet was resuspended in 1 ml cryopreservation medium, transferred into a cryovial and put on ice. To thaw the cryopreserved ovarian organoids, the cryovials were immediately thawed in a 37 °C water bath. Then, the contents were mixed with basic medium and centrifuged (2200 rpm, 5 min). Pellets were mixed with basic medium (15%) and Matrigel (85%). Subsequently, 20 µL droplets containing were dispensed into 48-well culture plates and incubated for 20 min. Finally, 250 µL of warm OvaOsCM supplemented with RI (10 µm) was added to each well. Culture media was changed every 3 days.

During ovarian organoid culture, bright-field microscope images were captured at 5x magnification to evaluate organoid growth. These images were subsequently analyzed using FIJI ImageJ software to calculate the total number of organoids and their average diameter.

Cell viability assays

The LIVE/DEAD Cell Imaging Kit (Thermo Fisher Scientific, R37601) was used to confirm ovarian organoid viability after passaging and thawing. According to the manufacturer's protocol, the same volume (150 µl) of

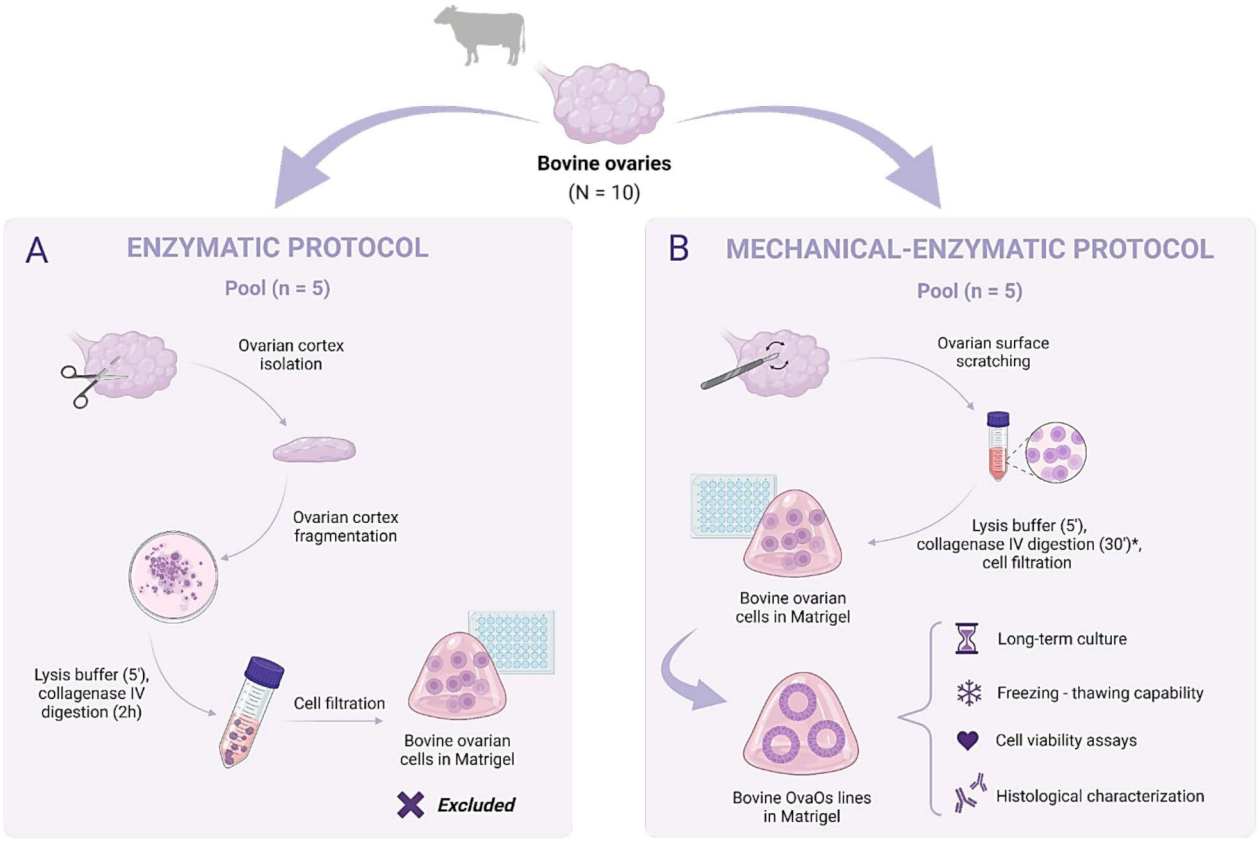


Fig. 4 Experimental design for bovine ovarian processing. Two different protocols were used: **(A)** Enzymatic protocol, which involves the fragmentation of the ovarian cortex followed by an intense digestion with collagenase IV; and **(B)** Mechanical-enzymatic protocol, based on scratching the ovarian surface to obtain OSE cells, with an optional soft step of collagenase IV digestion. Created with BioRender.com. *OvaOs: epithelial ovarian organoids. (*) This optional digestion step can be included if tissue aggregates remain present*

the 2X working solution was used for each well. After 15 min, PBS 1X was added to stop the reaction and samples were immediately examined under an inverted Zeiss Axio Vert.A1 microscope (Zeiss, Oberkochen, Germany) at ×5 and ×10 magnifications to assess cell viability. Cell viability was quantified by using FIJI ImageJ software (Rasband, USA).

Immunocytochemistry of bovine OSE cells and bovine OvaOs

For the fixation of bovine OSE cells and bovine OvaOs in Matrigel, a volume of 250 µl of 4% paraformaldehyde (PFA) (Sigma-Aldrich, 1.00496) was added to each well during 10 min at room temperature (RT). Then, all samples were permeabilized by using 250 µl of PBS 1X–Tween 0,05% during 5 min at RT. The blocking step was subsequently performed by adding of 250 µl of PBS 1X–Tween 0,05% - BSA 5% to each well during 1 h at RT. Finally, the primary antibody anti-CK18 was used (Antibodies, 2819R, 1:300) overnight at 4 °C, followed by the Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, A11034, 1:500) for 45 min at RT. All samples were counterstained with DAPI (Thermo Fisher Scientific, 62 248). Images were captured using a Nikon Eclipse 80i microscope (Nikon, Leuven, Belgium) and processed with FIJI-ImageJ software (Rasband, USA).

Histological characterization of bovine OvaOs

To dissolve the Matrigel drops and release the organoids, a volume of 250 µl of Vitrogel Organoid Recovery Solution (300k Solutions, MS04-10) was added to each well. Following this, OvaOs were transferred to an Eppendorf tube for centrifugation (600 g, 6 min), and the pellet was fixed with 4% PFA during 30 min at 4 °C. Three cycles of centrifugation and resuspension in 1 mL PBS 1X were performed before mixing the organoids with 200 µL HistoGel (Thermo Fisher Scientific, HG-4000-012) preheated to 55 °C. Samples were dehydrated in an increasing series of alcohols, embedded in paraffin, and cut into 5 µm sections. H&E staining were performed according to the manufacturer's protocol to visualize ovarian structures.

The expression and localization of CK18, VIM, and CD44 were assessed by immunofluorescence staining

Table 1 Composition of solutions and culture media used for bovine OvaOs generation, passaging, cryopreservation, and thawing. Concentrations and references of key components are provided to ensure reproducibility. *PBS: phosphate-buffered saline; P/S: penicillin-streptomycin; NH_4Cl: ammonium chloride; $NaHCO_3$: Sodium bicarbonate; DMEM F12: Dulbecco's modified eagle medium/nutrient mixture F-12; FBS: fetal bovine serum; A83-01: TGF-β receptor inhibitor; N2: N2 supplement; B27: B27 supplement minus vitamin A; EGF: epidermal growth factor; RSPO1: R-spondin 1; IGF1: insulin-like growth factor 1; HGF: hepatocyte growth factor; NRG1: neuregulin 1; RI: ROCK inhibitor; p38i: p38 MAP kinase inhibitor; DMSO: dimethyl sulfoxide*

Medium	Ingredients	Final concentration	Reference
Transportation and washing solution	PBS	1X	P3813
	P/S	0.1%	15,140,122
	FBS	10%	FBS-11 A
Lysis buffer	NH_4Cl	0,15 M	3,975,201
	EDTA	0,1 mM	E6758
	$NaHCO_3$	10 mM	S5761
	MiliQ H_2O	1X	1.01262
Basic medium	DMEM F12	1X	11,320,033
Ovarian organoids' culture medium (OvaOsCM)	DMEM F12	1X	11,320,033
	L-glutamine	1X	G7513
	P/S	1X	15,140,122
	A83-01	0.25 µm	9,094,360
	Nicotinamide	5 mM	N0636
	N2	1X	17,502,048
	B27 supplement minus vitamin A	1X	12,587,010
	N-acetilcysteine	1.25 mM	A9165
	17-β-estradiol	10 nM	E8875
	p38i (SB203580)	1 µm	SB203580
	EGF	50 ng/mL	AF-100-15
	Noggin	100 ng/mL	120-10c
	RSPO1	50 ng/mL	120–38
	IGF1	20 ng/mL	MSST0063
	HGF	10 ng/mL	100–39
	NRG1	50 ng/mL	492,027
	Y27632 (RI)	10 µm	688,001
Cryopreservation medium	DMEM F12	1X	11,320,033
	DMSO	10%	D8418

Table 2 Primary and secondary antibodies used for the histological characterization of bovine OvaOs. Concentrations and references of antibodies are provided to ensure reproducibility. *CK18: cytokeratin 18; VIM: vimentin; PAX8: paired box gene-8 protein*

Antibody	Marker/specificity	Cell localization	Dilution	Reference
Primary	CK18	Cytoplasm	1:300	2819R
	VIM	Cytoplasm	1:1000	Ab92547
	CD44	Cytoplasm	1:200	14–0441
	PAX8	Nucleus	1:1000	10336-1-AP
	Ki67	Nucleus	1:600	Ab15580
Secondary	Alexa Fluor 488-conjugated goat anti-rabbit IgG	-	1:500	A11034
	Alexa Fluor 568-conjugated goat anti-rabbit IgG1	-	1:500	A21124
	Goat anti-rabbit IgG	-	1:500	BA-1000
	Goat anti-Rat IgG (whole molecule)–FITC antibody	-	1:32	F6258

(Table 2). Organoids sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval with 10 mm sodium citrate buffer (pH 6) for 20 min in a 95 °C water bath. Samples were permeabilized with PBS-Tween 0.05% and blocked with PBS 1X– Tween 0.05% with 5% BSA for 1 h at RT. To detect CK18 and CD44 expression, organoids were incubated with anti-CK18 (Antibodies, 2819R, 1:300) and anti-CD44 (eBioscience™, 14–0441, 1:200) overnight at 4 °C, and followed by the Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, A11034, 1:500) and FITC-conjugated goat anti-rat IgG secondary antibody (Sigma Aldrich, 1:32, F6258), respectively (45 min at RT). Alternatively, VIM expression was evaluated by using

anti-VIM (Abcam, ab92547, 1:1000) for 30 min at RT, followed by its corresponding Alexa Fluor 568-conjugated goat anti-rabbit IgG1 cross-absorbed secondary antibody (Invitrogen, A21124, 1:500) incubated for 45 min at RT. All samples were counterstained with DAPI (Thermo Fisher Scientific, 62 248). Images were captured using a Nikon Eclipse 80i microscope (Nikon, Leuven, Belgium) and processed with FIJI-ImageJ software (Rasband, USA).

The expression of Ki67 and PAX8 was evaluated by immunohistochemistry (Table 2). After organoid deparaffination and rehydration, epitope retrieval was performed with Tris-EDTA buffer (pH 9). Samples were permeabilized with PBS 1X– Triton 0.01% and blocked with PBS-Tween 0.05% with 5% BSA for 1 h at RT. Subsequently, samples were incubated overnight at 4 °C with the primary antibody anti-Ki67 (Abcam, ab15580, 1:600) and anti-PAX8 (Proteintech, 10336-1-AP, 1:1000), respectively. After blocking endogenous peroxidase activity, slides were incubated with the secondary antibody goat anti-rabbit IgG (VectorLabs, BA-1000, 1:1500), followed by Vectastain ABC-HRP reagent (VectorLabs, PK-6100) and counterstained with hematoxylin. Positive and negative controls of these markers are included in the Supplementary Material section (Fig. 5, Supplementary Material).

Discussion

Organoid models have transformed bioengineering by providing advanced tools for replicating human tissues and modeling diseases in almost all medical disciplines. These 3D structures, derived from primary, progenitor or stem cells, offer controlled environments to study complex cellular interactions and tissue-specific responses in vitro [1–3]. In human reproductive biology, most of healthy and pathological organoids from the female reproductive tract are well-stablished and have demonstrated their value by mimicking tissue-specific architecture and responses [4, 5, 9, 24–26]. However, healthy human OvaOs remain significantly underexplored, underscoring their potential to advance research in ovarian physiology, folliculogenesis, and non-cancerous pathologies, such as primary ovarian insufficiency or polycystic ovarian syndrome [27].

In human ovarian bioengineering, many studies are focused on organoid models derived from ovarian cancers [11–13]. Most of these ovarian tumors, such as high-grade serous carcinoma, are predominantly epithelial, thus are mainly composed of epithelial cells [28]. This composition simplifies protocols for isolating OSE cells and generating epithelial organoids, as there is a greater abundance of target cells and less need for precise isolation methods. However, these cancer samples do not accurately reflect the cellular diversity and functional properties of healthy OSE tissue. In contrast, the physiological human OSE is a very thin membrane formed by a single layer of epithelial cells surrounding the ovary [16]. Due to its surface location, OSE in humans is highly exposed to the external environment, making it particularly vulnerable to damage during manipulation. This fragility complicates the adaptation of cancer-derived protocols to the healthy ovary [11]. Notably, some of these cancer studies include non-malignant OvaOs as controls, but the specific protocol for their derivation are often not detailed. This lack of methodological transparency highlights the need for detailed and reproducible protocols focusing on the generation of healthy OvaOs.

To our knowledge, recent studies have established protocols for murine [14] and human [15] OvaOs from fresh intact ovaries. Despite substantial efforts to enhance the efficiency of OvaOs culture through optimized media formulations [11, 15], the main handicap persists in the low availability of the samples and its delicate processing to obtain sufficient OSE cells for the OvaOs formation. In this context, our manuscript introduces key methodological differences to overcome these limitations. Regarding murine OvaOs, their derivation protocol rely exclusively on enzymatic digestion (pronase solution 1 mg/ml, 37 °C, 30 min) due to the small size of ovaries from mice [14]. However, this approach is difficult to extrapolate to bovine or human samples due to the larger size and increased complexity of mammalian ovaries. In fact, when we applied an exclusively enzymatic protocol, we were unable to obtain a sufficient yield of cells for organoid formation, even with higher enzyme concentrations (collagenase IV, 8 mg/ml) and extended enzymatic digestion (1–2 h). On the other hand, the human OvaOs protocol requires fresh intact human ovaries for initial enzymatic digestion followed by a mechanical scratching [15]. This method presents a significant limitation, as obtaining and processing these organs, whether from cadaveric donors or transgender patients, is logistically complex. Notably, our protocol, based on a first mechanical scraping followed by enzymatic digestion, can be also adapted to human ovarian cortex samples. We can selectively scratch the outer surface of the ovarian cortex– where the OSE is located– while preserving the inner surface in contact with the internal part of the ovary. This helps minimize the release of other ovarian cell types. Subsequent enzymatic digestion ensures the dissociation of OSE cell aggregates, facilitating the derivation and seeding of individualized OSE cells. Thus, our approach broadens the spectrum of viable samples for OvaOs derivation, making it feasible to work not only with fresh intact ovaries but also with ovarian cortex samples, which are more available in tissue banks.

In this study, we propose bovine ovaries as a valuable alternative for developing effective protocols to generate

healthy OvaOs. Bovine ovaries are abundant, biologically and genetically consistent, and share key structural and functional characteristics with human ovaries, including organ structure, ECM composition, and hormonal regulation [20, 29, 30]. In particular, the OSE from both species consists of a single layer of cuboidal epithelial cells that play structural and mechanical functions, including the maintenance of ovarian integrity and facilitation of follicular rupture during ovulation [16, 31]. Like in humans [23], bovine OSE cells are surrounded by an ECM enriched in collagens, hyaluronic acid, glycosaminoglycans, and fibronectin, among other proteins [22]. However, the collagen fiber network in the bovine ovarian cortex is less dense compared to human ovaries, which have smaller pore sizes and denser collagen fibers [21]. Despite these differences, we believe that our protocol can be adapted to human samples, as the use of an external ECM — in our case, Matrigel — compensates for variations in the native ECM composition. Furthermore, bovine and human ovarian hormonal regulation share fundamental similarities, particularly in the roles of GnRH, LH, FSH, estradiol and progesterone in controlling follicular development, ovulation, and luteolysis [21]. Because of these this, the culture medium used for human OvaOs [15], which includes hormones and growth factors, can also be applied to bovine OvaOs. All these similarities support the suitability of bovine ovaries as a model for developing and optimizing OvaOs culture before translating them to human ovarian bioengineering.

To successfully generate epithelial organoids from the ovary, cells must exhibit specific characteristics such as an epithelial nature, stem cell potential, and the ability to self-organize and differentiate into complex 3D cell structures [1]. In this context, OSE cells are particularly suitable candidates for generating these 3D models [32]. Here, two different procedures were tested for isolating these OSE cells, which included a purely enzymatic protocol and a mechanical-enzymatic method. The enzymatic protocol involved chemical disaggregation of the ovarian cortex but did not achieve sufficient OSE cell separation. This limitation may be attributed to the high levels of hyaluronic acid, collagen, and other extracellular matrix components that increase cortical rigidity [33], impeding effective cell release. Additionally, the exclusively enzymatic approach could lead to the isolation of other cell types along with OSE cells, potentially resulting in less pure organoid cultures. In contrast, the mechanical method yielded a greater number of OSE cells suitable for 3D culture. By scraping the outer surface of the ovary, this method allows for a more targeted selection of OSE cells [34]. Importantly, adding a brief collagenase digestion step following mechanical scraping facilitated the breakdown of OSE aggregations into single cells, significantly enhancing cell viability and organoid formation rates [35]. Unlike other approaches [15, 36], these OSE cells were directly seeded in Matrigel without prior expansion or cryopreservation, potentially reducing genetic instability and preserving their natural proliferative capacity [2].

The biological potential of bovine OvaOs, generated from the mechanical methodology, to establish long-term organoid lines was demonstrated by their exponential growth over time, passaging capacity, and high viability after freeze-thaw cycles. Initial organoid structures were formed by day 4 of culture, with their epithelial origin confirmed by positive CK18 expression. As culture progressed, bovine OvaOs showed sustained growth, reflected by increases in both organoid diameter and total number, indicating active cellular proliferation and structural maturation. However, the total organoid count began to decrease in larger stages, as OSE cells prioritized the expansion of existing organoids over generating new structures. Notably, the diameter of our bovine OvaOs was very similar to that from human healthy OvaOs described in the literature [15]. By day 21, the high cellular density within Matrigel drops, combined with the inherent softness of the matrix, required passaging (or freezing) to maintain cell viability. This outcome aligns with findings in similar studies [11, 15], where the mechanical limitations of tumor-derived matrices (Matrigel®, Cultrex®) difficult OvaOs culture beyond 21 days per passage. Despite these challenges, bovine OvaOs in Matrigel consistently reformed and proliferated after each passage or freeze-thaw cycle, likely due to the high cell viability (> 90%) observed following these processes. However, the low viscosity and the malignant origin of tumor-derived scaffolds highlight the need to explore alternative natural or hybrid biomaterials, such as decellularized tissue-derived hydrogels. These alternatives can provide biochemical cues from native tissue — rather than a cancerous microenvironment — to the organoid culture [22, 37, 38].

Histological analyses confirmed that bovine OvaOs closely resemble native OSE, evidenced by the expression of key markers including CK18, VIM, and CD44. Ensuring the maintenance of this expression profile in organoid models, which parallels that found in the human ovary [16], is critical for accurately recapitulating the original tissue and enhancing the fidelity of the model. The co-expression of CK18 and VIM in bovine OvaOs underscores their epithelial-mesenchymal properties inherent to OSE cells [39, 40], which are involved in ovarian functions such as post-ovulatory wound healing and tissue remodeling. In addition, OSE cells from the bovine OvaOs expressed CD44, a key transmembrane protein involved in many cell-cell and cell-matrix interactions in the ovarian cortex [41]. CD44 primarily binds

to hyaluronic acid, a key component of the extracellular matrix, but also interacts with osteopontin, serglycin, collagens, fibronectin, and laminin. These interactions are critical for maintaining tissue structure and facilitating cell migration [42]. In addition to its role in adhesion and migration processes, CD44 is also described as a marker for ovarian stem/progenitor cells [15, 16]. The presence of these cells in organoid structures has been previously reported in other female tissues, such as the endometrium [43–45], and contributes to their proliferative, self-renewal, and regenerative potential. Notably, immunohistochemistry also revealed an absence of PAX8 protein expression, which is typically associated with fimbrial cells [46]. Although previous studies have been reported discrepancies in PAX8 expression of the OSE tissue [19], we observed PAX8 nuclear signal exclusively in cells from inclusion cysts within the ovarian cortex, which, based on their marker profile ($PAX8^+$, $WT1^+$, $calretinin^-$ and $D2\text{-}40^-$), are consistent with a phenotype resembling ciliated tubal epithelium [47]. This result aligns with the widely accepted view that OSE cells do not express this marker [48, 49], further supporting the phenotypic distinction between OSE and these inclusion cysts.

Among its applications, the culture of bovine OvaOs offers a valuable tool for developing more complex cellular models that better recapitulate the architecture of the ovarian cortex. These next-generation cultures, commonly referred to as assembloids, have already been successfully established in other parts of the female reproductive tract, such as the endometrium [50]. To achieve this, additional ovarian cell populations, particularly stromal cells, must be incorporated in specific proportions at the time of OvaOs seeding in Matrigel. This strategy would enable proper cell-cell interactions and spatial organization, closely resembling the native ovarian microenvironment. Moreover, these advanced models would allow functional studies, including hormonal stimulation. In contrast to other organoid models [4], OvaOs alone exhibit limited endocrine responsiveness, as gonadotropin supplementation induces only a slightly increase in their number and size [15]. Ultimately, by optimizing these assembloids, they could serve as a platform for co-culturing with ovarian follicles, which include granulosa and theca cells, further enhancing their physiological relevance and applicability in reproductive research.

This study has several limitations. First, the functional capacity of bovine OvaOs remains unexplored, requiring further model refinement through the inclusion of additional ovarian cell types before evaluating hormonal responsiveness and follicle support. Additionally, the three-dimensional scaffold could be improved because the tumorigenic origin and 14-day in vitro stability of Matrigel. The use of natural or hybrid hydrogels may provide a more biomimetic microenvironment with extended culture viability. Finally, validation in human ovarian samples, particularly ovarian cortex tissues, is essential to optimize the OvaOs derivation protocol beyond fresh intact human ovaries.

Taken together, this derivation technique for bovine OvaOs provides critical insights for the generation of human OvaOs. As highly customizable 3D models, OvaOs hold the potential for co-culture systems that facilitate comprehensive exploration of cell interactions within the female reproductive tract [50, 51]. Moreover, the OvaOs culture in alternative 3D scaffolds with enhanced mechanical properties, such as hybrid hydrogels, may improve structural support and enable prolonged assays [37]. By refining and adapting this protocol to human samples, OvaOs represent the first step to bioengineering more complex models, with future possible applications in fertility preservation, regenerative medicine, or disease modelling [52].

Conclusion

In conclusion, this study describes an effective protocol based on mechanical-enzymatic methodology for deriving healthy bovine OvaOs as a physiological model for ovarian bioengineering. These bovine OvaOs successfully replicate key properties of the bovine OSE ($CK18^+$, VIM^+, $CD44^+$, $PAX8^-$), achieving high viability over long-term culture, passaging, and freeze-thaw cycles. Future research should focus on co-culturing OvaOs with other ovarian cell types or follicles to better assess their functional relevance, including hormone secretion and follicular support. Once these advances are established, the model can be directly adapted to human ovarian tissue, given the biological similarities between bovine and human ovaries.

Supplementary Information

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Supplementary Material 1

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Author contributions

Conception of the work: M.G.Á., I.C., and A.P.; design of the work: M.G.Á and I.C.; acquisition, analysis, and interpretation of data: M.G.Á., E.F.H., M.A.H., C.B.F., P.A.F., N.C., and A.F.; manuscript and figure preparation: M.G.Á.; manuscript review: I.C., E.F.H., M.A.H., C.B.F., P.A.F., N.C., and A.P. All authors have approved the submitted version. All authors approve the submitted version and agree both to be personally accountable for the author's own contributions, and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved, and the resolution documented in the literature.

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Data availability
No datasets were generated or analysed during the current study.

Code availability
Not applicable

Declarations

Ethics approval and consent to participate.
Not applicable

Consent for publication
Not applicable

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