Ovarian extracellular matrix-based hydrogel for human ovarian follicle survival in vivo: A pilot work

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Abstract
To successfully assemble a bio-engineered ovary, we need to create a three-dimensional matrix able to accommodate isolated follicles and cells. The goal of this study was to develop an extracellular matrix hydrogel (oECM) derived from decellularized bovine ovaries able to support, in combination with alginate, human ovarian follicle survival and growth in vitro. Two different hydrogels (oECM1, oECM2) were produced and compared in terms of decellularization efficiency (dsDNA), ECM preservation (collagen and glycosaminoglycan levels), ultrastructure, rigidity, and cytotoxicity. oECM2 showed significantly less dsDNA, greater retention of glycosaminoglycans and better rigidity than oECM1. Isolated human ovarian follicles were then encapsulated in four selected hydrogel combinations: (1) 100% oECM2, (2) 90% oECM2 + 10% alginate, (3) 75% oECM2 + 25% alginate, and (4) 100% alginate. After 1 week of in vitro culture, follicle recovery rate, viability, and growth were analyzed. On day 7 of in vitro culture, follicle recovery rates were 0%, 23%, 65%, 82% in groups 1–4, respectively, rising proportionally with increased alginate content. However, there was no difference in follicle viability or growth between groups 2 and 3 and controls (group 4). In conclusion, since pure alginate cannot be used to graft preantral follicles due to its poor revascularization and degradation after grafting, oECM2 hydrogel combined with alginate may provide a new and promising alternative to graft isolated human follicles in a bio-engineered ovary.

KEYWORDS
bio-engineered ovary, ECM hydrogels, ovarian decellularized extracellular matrix, ovarian follicles

1 | INTRODUCTION

In some cancers grafting of frozen/thawed ovarian tissue cannot be performed due to the risk of reintroducing malignant cells. An alternative to restore fertility in these patients is development of a transplantable engineered ovary (TEO). It consists of isolated ovarian follicles, the morpho-functional units of the ovary, which are co-encapsulated with isolated ovarian cells in a three-dimensional (3D) scaffold, essential to providing a bio-engineered environment where follicles can be supported, grow, and interact with ovarian cells.
Encouraging results with mouse follicles have been obtained in vitro and in vivo with alginate scaffolds. While alginate has several advantages for follicle encapsulation, one of the main limitations is its biological inertness, which has hampered its use in vivo applications due to poor cell adhesion, limited degradation and revascularization after transplantation. To circumvent this problem, previous studies with mouse follicles aimed to combine alginate with other natural polymers. Despite their promising results, alginate-based hydrogels still lack many bioactive factors present in the ovarian extracellular matrix (ECM). To develop a 3D scaffold as similar as possible to the native organ, hydrogels from decellularized ECM have been proposed. Decellularization of mammalian tissues aims to remove antigenic components, while maintaining the biochemical composition of native ECM. Decellularized tissues can be comminuted to form ECM powders that, once digested and solubilized into constituent protein monomers, can be further pH- and temperature-neutralized to induce spontaneous reformation into a hydrogel. ECM hydrogels are collagen-based and enriched with native sulphated glycosaminoglycans (GAGs), proteoglycans, and ECM proteins. When processed appropriately, ECM hydrogels retain the inherent bioactive features of native ECM, including peptide domains, which play a major role in integrin-cell attachment, cell survival, proliferation, and deposition of de novo ECM components. Moreover, they can be easily administered in a minimally invasive manner. The thermoresponsive nature of ECM hydrogels allows injection of liquid-like, solubilized ECM that will form a scaffold at physiological body temperatures. However, one of the limitations of pure ECM-derived hydrogels is their low mechanical properties and rigidity which has led to combinations with other polymers. Tissue-derived ECM hydrogels have already demonstrated their potential in a variety of different tissues and organs, including heart, brain, bone, skin, intestine, liver, kidney, meniscus, and spinal cord.

Since each ECM is unique in its topological, mechanical, and biochemical profile, one of the major challenges in developing ECM-derived hydrogels is tailoring tissue-specific decellularization protocols able to preserve the biochemical and physical characteristics of native ECM. Xenogeneic ECM-derived scaffolds are already being tested in increasing numbers of preclinical trials, and no cases of zoonosis have been reported to date. Moreover, better outcomes were achieved in terms of efficacy and reproducibility when animal-derived ECMs were compared with human-derived ECM. Importantly, the feasibility and safety of animal-derived ECM hydrogels have recently been documented in human clinical trials. These important studies open up the possibility of using animal-derived ECM hydrogels in many other applications.

The goal of this pilot study was to develop and characterize a hydrogel derived from decellularized bovine ovarian extracellular matrix (oECM) to support survival and development of isolated human follicles when it was combined with alginate. Bovine ovaries were chosen due to their similarity to human ovaries in terms of ultrastructure, follicle dynamics, rigidity, ECM organization, and composition and unlimited supply, since they are normally discarded. Furthermore, some bovine-ECM products have already been commercialized and reportedly used without any safety issues which is promising for extending possible clinical applications.

In this work, two different decellularization protocols were tested in order to assess their impact on the hydrogel properties and ECM preservation: the first protocol had a prolonged enzymatic treatment, and the second had a short enzymatic treatment in combination with detergents. As bovine ECM-derived hydrogels (oECM) have not previously been used to encapsulate isolated ovarian preantral follicles, we first evaluated hydrogel impact on mouse and human follicle viability. Then to obtain mechanical properties closer to the human ovary, the selected oECM hydrogel was combined with alginate to increase its stiffness.

2 MATERIALS AND METHODS

2.1 Ethics

The Institutional Review Board of the Université Catholique de Louvain approved the use of human ovarian tissue to isolate preantral ovarian follicles for this study (reference 2012/23 mar/125). Guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain on June 29, 2016 (reference 2014/UCL/MD/007).

2.2 Chemicals and reagents

All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) unless otherwise stated.

2.3 Bovine ovary-based ECM hydrogel (oECM)

Bovine ovaries (n = 2) were obtained from young animals (aged 8–10 years old) from a local slaughterhouse (Abattoir d’Anderlecht, Brussels, Belgium), transported in Dulbecco’s phosphate-buffered saline (DPBS) at 4°C, and processed within 1 h. A longitudinal incision was made to remove the medulla, all the antral follicles were aspirated, and corpora lutea were removed. The tissue was cut into 1 cm² fragments, plunged into liquid nitrogen for immediate freezing, and stored at −80°C until further use. The choice of decellularization protocol depends on tissue-specific factors, including cellularity and density, with most protocols utilizing chemical, physical or enzymatic agents. In this study, ovaries were decellularised using enzymatic agents alone (protocol 1) or with detergents (protocol 2) (Figure 1).

2.3.1 Decellularization of ovarian tissue: protocol 1 (enzymatic)

Thawed ovarian fragments were washed thoroughly in deionized water and incubated in a 0.05% trypsin-0.02% EDTA solution for 24 h at 37°C and shaken at 300 rpm. The following day, the fragments were washed in DPBS (1×) and then incubated in DPBS for 24 h at room temperature (RT) under agitation. After a final wash in deionized water, decellularized ovarian fragments were freeze-dried.
2.3.2 | Decellularization of ovarian tissue: protocol 2 (enzymatic + detergents)

Thawed ovarian fragments were washed thoroughly in deionized water and incubated in a 0.05% trypsin-0.02% EDTA solution for 1 h at 37°C and shaken at 300 rpm. The material was then washed in deionized water, incubated in 3% Triton X-100 for 1 h at RT agitated, washed again in deionized water, and incubated in 4% sodium deoxycholate under agitation for 1 h at RT. After a final wash with deionized water, the tissue was stored in water overnight at 4°C and then incubated in 4% ethanol and 0.2% peracetic acid for 2 h at RT under agitation before being washed thoroughly in DPBS and then deionized water before freeze-drying.

2.3.3 | ECM digestion and solubilization

Pepsin digestion and solubilization were carried out to obtain pre-gel solutions. Briefly, lyophilized ECM was added to 1 mg/mL of pepsin in 0.01 N hydrochloric acid (HCl) for a final concentration of 10 mg ECM/mL. The suspension was stirred at RT for 72 h. The resulting pepsin digests were aliquoted and stored at −20°C. Gelation of pepsin digests was induced by neutralization of salt concentrations and pH at 4°C, followed by warming to 37°C. Pre-gel solutions of 8 mg/mL were prepared and incubated for 1 h at 37°C to obtain solid hydrogels according to each protocol, designated as oECM1 and oECM2.

2.4 | oECM characterization

2.4.1 | DNA, collagen, and sulphated glycosaminoglycan quantification

More detailed protocols are described in the Supplementary file.

Determination of decellularization efficiency was based on quantification of dry weight double-stranded DNA (dsDNA). Concentrations of dsDNA were measured using a Quant-it™ Picogreen® assay kit (Invitrogen, Merelbeke, Belgium) following the supplier’s instructions.

Since collagen and sulphated GAGs are structural ECM proteins, their content was investigated to determine the rate of ECM preservation. Collagen content was measured by quantification of hydroxyproline. Briefly, bovine ovarian tissue, oECMs, and a blank pepsin solution were treated in the same way. The hydrolyzed blank pepsin solution served as a negative control as well as a diluent for the assay. A standard curve was prepared using known concentrations of hydroxyproline, ranging from 0 to 100 μg/mL, and absorbance was measured at 540 nm with a Tecan Infinite M200 Plate reader. Sulphated GAGs were determined using the 1,9 dimethyl-methylene blue (DMMB) assay, while a blank pepsin solution served as a negative control as well as a diluent for the assay. A total of 50 μL of samples were mixed with 200 μL of DMMB solution (0.03 M sodium formate, 0.046 M DMMB, ethanol [0.5% v/v] and formic acid [0.2% v/v] in a 96-well plate. The standard curve ranged from 0 to 75 μg/mL chondroitin-4-sulphate. Absorbance was measured immediately at 525 nm.

2.4.2 | Mechanical properties of oECM hydrogel

Rheological and gelation characteristics of oECM1 and oECM2 were determined as previously described using a Malvern Kinexus ultra+ rheometer (Malvern Kinexus Pro rotational rheometer and rSpace software, Malvern Instruments, Worcestershire, UK). Data were recorded every 30 s (n = 3).

2.4.3 | Microstructure of oECM hydrogel

Both oECM1 and oECM2 (300 μL per well) were fixed in 5% glutaraldehyde at RT. Samples were dehydrated in a graded series of ethanol (25–100%), dried using a critical point dryer (Balzers Union, Balzers, Finland), and sputter-coated with gold using a Cressington 208HR metalizer (Elodie s.a.r.l., Tremblay, France). Images were acquired using a JSM-7600F (JEOL, Zaventem, Belgium) scanning electron microscope at a voltage of 15 kV. Bovine ovarian tissue was used as a control.
2.5 | Isolation and viability of preantral follicles

2.5.1 | Isolation and viability of mouse preantral follicles

All procedures were carried out under a laminar flow hood using sterilized materials. The ovaries of four severe combined immunodeficient (SCID) mice (5 weeks of age) were removed, as already reported, and follicles were isolated according to our routine procedure. To assess the impact of the follicle isolation procedure, follicle viability was evaluated soon after isolation using a vital assay (Invitrogen), as previously described. Follicles were classified into four categories based on oocyte and granulosa cell viability: V1 and V2 represented follicles with high viability (0% and <10% dead granulosa cells, respectively) and V3 and V4, follicles with poor viability (≥10% and >50% dead granulosa cells or a dead oocyte). Before encapsulation, follicles were first washed and then embedded into oECM1 and oECM2.

2.5.2 | Isolation and viability of human preantral follicles

Frozen ovarian tissue samples from three patients of reproductive age with non-ovarian pathologies were taken from the ovarian tissue bank, after obtaining written informed consent. Tissues were thawed and follicles isolated. Follicle viability was calculated as described above (see section 2.5.1).

2.6 | Ovarian follicle encapsulation

2.6.1 | Mouse ovarian follicle encapsulation

Mouse ovarian follicles were encapsulated only in oECM1 and oECM2 to evaluate the cytotoxicity of the ovarian ECM hydrogels and avoid any waste of precious human follicles.

Firstly 339 follicles were divided into two groups (oECM1 and oECM2), and their diameter was recorded before being encapsulated separately in each group. Between 10 and 15 follicles were embedded in 50 μL oECM1 (8 mg/mL) or oECM2 (8 mg/mL), placed in a 4-well plate and incubated at 37°C for 1 h to allow gelation (oECM1 n = 20 beads, oECM2 n = 3 beads). Thereafter, 500 μL pre-equilibrated in vitro culture medium (MEM, GlutaMAX, Gibco, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (Gibco), 2 mM pyruvic acid 50 μg/mL ascorbic acid, 0.1 IU/mL follicle stimulating hormone (FSH) (Gonal F, Merck, Darmstadt, Germany), 1% Antibiotic-Antimycotic (Anti-Anti; Gibco) were added. The hydrogels were cultured at 37°C and 5% CO2 for 1 week. Every 2 days, half of the culture medium was removed and replaced with fresh medium.

2.6.2 | Human ovarian follicle encapsulation

A more detailed protocol is described in the Supplementary file.

Isolated primordial and primary follicles (n = 114) were divided into 4 groups and their diameter was recorded before being encapsulated in: (1) 100% oECM2 (8 mg/mL); (2) 90% oECM2 + 10% of 1% alginate (SLM100; Pronova, Nova Matrix, Sandvika, Norway); (3) 75% oECM2 + 25% of 1% alginate; or (4) 100% of 1% alginate. Incubation at 37°C for 1 h was performed to allow oECM2 to gel, which was then followed by addition of 100 μL CaCl2 100 mM for a few seconds to induce alginate cross-linking. A 100 μL of culture medium was then added to each well and the samples were stored at 37°C and 5% CO2 for 1 week. Every 2 days, half of the culture medium was removed and replaced with fresh medium.

2.7 | Follicle recovery rate, viability, and in vitro growth

Follicle recovery rates, viability and growth were evaluated after 7 days of in vitro culture. On day 7, the follicle recovery rate was calculated for each bead and then compared (mean ± SD) for each group as follow:

\[
\text{Number of recovered follicles} = \left( \frac{F_E}{F_F} \right) \times 100
\]

where \( F_E \) is the encapsulated follicles on day 0; \( F_F \) is the follicles found on day 7.

![Figure 2](image_url) Influence of the ovarian decellularization protocol on dsDNA, collagen and GAG content Efficiency of decellularization was assessed by quantifying level of dry weight dsDNA/mg (A). Preservation of the ECM after decellularization was assessed by quantifying collagen (B) sulphated GAG (C) and rheological properties (D) through investigation of storage (\( G' \)) and loss moduli (\( G'' \)) Data are presented as mean ± SD. Significant differences are represented by asterisks. (** p < .01; ****p < .0001). Each group was analysed in triplicate.
In order to measure follicle viability, vital assays were conducted as previously documented (see section 2.5.1).

Follicle growth was evaluated by measuring follicle diameters under an inverted microscope, using a scale incorporated in the objective lens at 10× magnification. On days 0 and 7 of in vitro culture, each follicle was measured and the mean ± SD was compared between these time points for each group.

### Statistical analysis

Statistical analyses were performed with Prism 8.1.2. Detailed methods are described in the Supplementary file. This research has benefited greatly from statistical consultation with the Statistical Methodology and Computing Service technological platform at the UCL (SMCS/IMMAQ, UCL).

### RESULTS

#### Decellularization impacted residual DNA and GAGs content, but not collagen proportions in oECM hydrogels

Table 1 presents the storage and loss modulus (mean ± SD) in oECM1 and oECM2 hydrogels. The concentration of nucleic acid (mean ± SD) was significantly higher \((p < .0001)\) in native tissue (1750-fold) than in oECM1 or in oECM2 (Figure 2A).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Storage and loss modulus (mean ± SD) in oECM1 and oECM2 hydrogels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oECM1 (n = 3)</td>
</tr>
<tr>
<td>Storage modulus ((G'))</td>
<td>65 ± 18 Pa(^a)</td>
</tr>
<tr>
<td>Loss modulus ((G')')</td>
<td>9.5 ± 2 Pa(^a)</td>
</tr>
</tbody>
</table>

Note: Different superscript letters indicate statistical differences \((a, b, p < .05)\).

Abbreviations: oECM1, ovarian-derived extracellular matrix hydrogel 1. oECM2, ovarian-derived extracellular matrix hydrogel 2.

#### FIGURE 3

Influence of decellularization on oECM hydrogel microstructure

Native bovine ovarian tissue (A), oECM1 (B) and oECM2 (C) hydrogels were processed for scanning electron microscopy. Magnification: 12,000×; scale bar: 1 μm

#### FIGURE 4

Influence of oECM hydrogel on mouse ovarian follicle morphology

Isolated mouse preantral follicles (from primordial-to secondary follicle stage) before encapsulation in oECM1 (A) and oECM2 (B) hydrogels. No difference in follicle morphology was found (A, B) before in vitro culture. Mouse follicles in oECM1 on day 7 of in vitro culture (C), mouse follicles in oECM2 on day 7 of in vitro culture (D). Black arrow indicates encapsulated ovarian follicles.
When comparing oECM1 and oECM2, a significantly \( p < .0001 \) higher concentration of dsDNA was found in the oECM1 hydrogel.

No difference in collagen content was observed between native tissue \( (n = 2, 0.67 \pm 0.3 \, \text{mg/mg of dry weight}) \), oECM1 \( (n = 3, 0.89 \pm 0.06 \, \text{mg/mg of dry weight}) \) or oECM2 \( (n = 3, 0.67 \pm 0.2 \, \text{mg/mg of dry weight}) \) (Figure 2B). sGAG content was significantly higher \( (p < .01) \) in oECM2 (2.7-fold) compared to oECM1 and equivalent to native tissue (Figure 2C).

3.2 | Decellularization influenced oECM hydrogel mechanical properties

Storage modulus \( (G') \) and the loss modulus \( (G'') \) represent the mechanical strength and the viscous portion of hydrogel, respectively. \( G' \) and \( G'' \) of pre-gel solutions increased after 5 min after pH neutralization and temperature elevation (Figure 2D), indicating the beginning of the gelation process that plateaued after 8 min. \( G' \) was always higher than \( G'' \) in both hydrogels. Irrespective of the decellularization protocol, gelation kinetics were similar, even though the final moduli were statistically different (Table 1). Indeed, oECM2 showed a \( G' \) value superior \( (p < .05) \) to the oECM1 hydrogel modulus, while oECM1 hydrogel \( G'' \) was significantly higher \( (p < .0001) \) than the oECM2 hydrogel.

**TABLE 2** Mouse ovarian follicle diameter (mean ± SD) in oECM1 and oECM2 on day 0 and day 7 of in vitro culture

<table>
<thead>
<tr>
<th>Mouse follicle diameter (mean ± SD)</th>
<th>oECM1</th>
<th>oECM2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>45 ± 22 µm(^a) ((n=235))</td>
<td>51 ± 27.5 µm(^b) ((n=54))</td>
</tr>
<tr>
<td>Day 7</td>
<td>43 ± 21 µm(^a) ((n=174))</td>
<td>65 ± 34 µm(^b) ((n=12))</td>
</tr>
</tbody>
</table>

Note: Different superscript letters indicate statistical differences \( a, b, p < .05 \). Abbreviations: oECM1, ovarian-derived extracellular matrix hydgdgel 1. oECM2, ovarian-derived extracellular matrix hydgdgel 2.

3.3 | Influence of decellularization on oECM hydrogel microstructure

Bovine ovarian tissue was structurally heterogeneous. Dense and compact regions alternated with fibrous regions showing fibers of variable thickness. The porosity of the bovine ovarian ECM appeared to correlate inversely with the degree of crosslinking of tissue fibers. Regions with thicker tissue fibers and apparently inferior porosity alternated with regions of thinner fibers and greater porosity (Figure 3A). Decellularization of oECM hydrogels strongly influenced their morphology (Figure 3). While the oECM1 hydrogel had a highly porous and fibrillary structure with small and homogeneous fibers (Figure 3B), oECM2 hydrogel displayed a sponge-like structure with thicker walls and heterogeneous pores (Figure 3C).

3.4 | Impact of decellularization on follicle recovery, viability, and growth

3.4.1 | Mouse follicle recovery rate, viability and growth

A total of 339 mouse follicles were isolated under the stereomicroscope. Before follicle encapsulation and in vitro culture, 50 follicles were investigated for follicle viability. Percentages \( (n = 50 \text{ follicles}) \) of fully viable \( (V1) \) and minimally damaged \( (V2) \) follicles were 22% and

**TABLE 3** Human ovarian follicle viability (mean ± SD) in oECM2-based hydrogels on day 7 of in vitro culture

<table>
<thead>
<tr>
<th>Human ovarian follicle viability (V1 + V2)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*ND</td>
<td>87.5%(^a)</td>
<td>97.2%(^a)</td>
<td>87.5%(^a)</td>
</tr>
</tbody>
</table>

Note: Different superscript letters indicate statistical differences \( a, b, p < .05 \). Group 1: 100% oECM2 hydrogel. Group 2: 90% oECM2 hydrogel + 10% alginate. Group 3: 75% oECM2 hydrogel + 25% alginate. Group 4: 100% alginate.

*ND: no follicles were found in oECM2 on day 7.

**FIGURE 5** Influence of oECM2- hydrogel combinations on human ovarian follicle morphology. Group 2 represents 90% oECM2 + 10% alginate bead (A). Group 3 represents 75% oECM2 + 25% alginate bead (B). Group 4 represents 100% alginate bead. Black arrows indicate the presence of human follicles on day 7 of in vitro culture (A – C). When alginate was combined to oECM2, the hydrogel bead appeared more transparent and homogenous in a dose dependent manner after 7 days of in vitro culture.
TABLE 4 Human ovarian follicle diameter (mean ± SD) in boECM2-based hydrogels on day 0 and day 7 in vitro culture

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 7 *ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33 ± 20 μm^a (n = 12)</td>
<td>38 ± 12 μm^a (n = 15)</td>
</tr>
<tr>
<td>2</td>
<td>43 ± 12 μm^a (n = 15)</td>
<td>59 ± 24 μm^b (n = 8)</td>
</tr>
<tr>
<td>3</td>
<td>58 ± 24 μm^b (n = 12)</td>
<td>58 ± 19 μm^b (n = 17)</td>
</tr>
<tr>
<td>4</td>
<td>38 ± 12 μm^a (n = 20)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Different superscript letters indicate statistical differences (a,b p < .05). Group 1:100% oECM2 hydrogel. Group 2:90% oECM2 hydrogel +10% alginate. Group 3:75% oECM2 hydrogel +25% alginate. Group 4:100% alginate. *ND: no follicles were found in oECM2 on day 7.

52%, respectively, while highly damaged (V3) or dead (V4) follicles represented 10% and 16%, respectively. Hence, 74% of follicles were viable soon after isolation from mouse ovaries.

After encapsulation in oECM hydrogels and 1 week of culture, the follicle morphology was similar between oECM1 and oECM2 (Figure 4). Follicle recovery rates (54 ± 27% oECM1 [n = 20 beads], 47.5 ± 39% oECM2 [n = 3 beads]) and viability (V1 + V2, 69.5% and 50%) were not statistically different. Regarding follicle diameter, no statistical difference was observed regardless of oECM hydrogels used between day 0 and day 7 (Table 2).

3.4.2 Human follicle recovery rate, viability, and growth

For experiments with human follicles, only oECM2 was selected, based on its lower dsDNA content, higher GAG content and higher storage modulus. A total of 127 human follicles were identified under the stereomicroscope. Percentages of fully viable (V1) and minimally damaged (V2) follicles were 61% and 31%, respectively, which means that 92% of isolated follicles were viable before follicle encapsulation. No highly damaged (V3) and very few dead (V4) follicles (8%) were counted before follicle encapsulation.

On day 7, follicle morphology was similar between all groups but one (group 1, no follicle found) (Figure 5). Follicle recovery rates increased proportionally with the percentage of alginate added to oECM2: 0%, 23%, 65%, and 85% from group 1 to group 4, respectively. No follicles were recovered in group 1, but granulosa cells were attached and spread all over the hydrogel beads. After 1 week of culture in the different hydrogels, no difference was observed in follicle viability (Table 3) in any but one (group 1). Additionally, follicle diameter was similarly and significantly increased in any but one (group 1) (Table 4).

4 | DISCUSSION

In this study, two different ECM-derived hydrogels were produced from bovine ovaries. When decellularization efficiency was investigated, both hydrogels showed a drastic reduction in dsDNA compared to native tissue. According to Crapo et al. decellularized hydrogels should possess less than 50 ng dsDNA per mg of initial dry weight of decellularized ECM to limit the presence of residual antigenic material, which may induce an immune response in vivo. Although both hydrogels contained less than 50 ng/mg dsDNA, a significantly greater reduction in dsDNA (>99%) was obtained with the second protocol (oECM2) compared to native tissue. One of the main reasons for this difference is likely the addition of ionic (Triton X-100) and non-ionic (sodium deoxycholate) detergents to the second decellularization protocol. Indeed, Triton X-100 is known to disrupt DNA-protein interactions, and sodium deoxycholate to efficiently disrupt cell nuclei and membranes. Compared to other protocols for decellularization of mouse, bovine, porcine and even human ovaries, we achieved a greater reduction in initial dsDNA without using DNase, which has been shown to induce cytotoxicity of reseeded cells. Quantification of collagen and GAGs, major structural components of the ECM, provided assessment of ECM preservation in our oECM hydrogels. Similarly to other studies, we did not observe any difference in collagen content between native tissue and either of the two hydrogels. However, only protocol oECM2 allowed preservation of similar levels of GAGs to native tissue. GAGs are heteropolysaccharides, not only involved in ECM structure, but also in water retention and growth factor sequestering. GAGs can thereby promote cell adhesion, growth, differentiation and migration. A recent proteomic study on human ovarian tissue demonstrated that GAGs are widely distributed throughout human ovarian cortex, making them key components of the native ovarian ECM. Several authors have also shown that GAGs are directly or indirectly involved in follicle development, granulosa cell differentiation, and cumulus-oocyte expansion. Hence an enriched GAG hydrogel, such as oECM2 may better support human follicle survival in vitro than oECM1.

Physical scaffold properties, such as hydrogel rigidity and nanostructure, are other essential parameter for the creation of a TEO. As expected, both oECM1 and oECM2 exhibited a lower storage modulus than native ovarian tissue (~3.5 KPa), but more interestingly, they differed significantly from each other, with oECM2 being more rigid than oECM1. Because both hydrogels were derived from the same tissue source, with equal concentration and similar collagen content, the difference in stiffness is likely due to either the higher numbers of sulphated GAG retained in oECM2 and/or different ultrastructure observed by scanning electron microscopy. Structurally, oECM2 exhibited a less fibrous architecture with thicker and more...
compact walls than oECM1, which may reflect a distinct collagen fiber reorganization during gelation and hence a different degree of rigidity.

It could also be the result of the shorter enzymatic decellu-
larization process in the second protocol, which in turn better preserves GAG concentration and generated larger fibers.

Although the ultrastructure of oECM1 might appear more similar than oECM2 to the human ovary, additional investigations are nec-

essary before uttering any conclusions. Since all the other parameters of oECM1 (DNA content, GAGs, and storage modulus) were statistically inferior to oECM2, given a possible clinical application, a safer biomaterial with inferior cellular debris, like oECM2, is the first choice. To assess hydrogel cytocompatibility, isolated mouse preantral follicles were initially used in order to safeguard precious human follicles.

No statistical differences were found in follicle recovery rates or viability between oECM1 and oECM2 after 1 week of in vitro culture, so both hydrogels were equally able to support mouse follicle survival in vitro. However, since the aim of this study was to create a support-

ive scaffold for human ovarian follicles, it is important to bear in mind that the follicle requirements of a TEO are species-specific and differ substantially between rodents and humans.

Therefore, based on the lower dsDNA content, greater GAG retention, and superior stiffness, which are all favorable conditions to better support human follicle survival, we selected oECM2 as a promising new scaffold material for the TEO, using it to encapsulate and culture human follicles. In contrast to what we observed with fresh mouse follicles, no human follicles were found in the oECM2 alone after 1 week of in vitro culture. However, fully viable and elongated granulosa cells (as evidenced by live/dead assay) were present throughout this hydrogel. These different findings may be due to suboptimal in vitro culture conditions for isolated human primordial/primary follicles and the cryopreservation procedure applied to human ovarian tissue. Indeed, Wang et al. demonstrated that freshly isolated human ovarian follicles were less impaired in terms of viability and growth than vitrified and slow-frozen counterparts after 8 days of in vitro culture. Additionally, this difference may also highlight that mouse and human follicles require different environments and that decellularized ovarian ECM hydrogels may not be able to uniformly support species-

independent follicle survival and growth.

Considering the impact of matrix rigidity on human follicle survival, and because the oECM2 modulus was lower than in

native ovary, we decided to increase the mechanical properties of the ECM hydrogel by adding alginate. As expected, when alginate was combined with oECM2, the follicle recovery rates increased proportionally, up to a maximum achieved with alginate alone. In 7-day cul-
tures of oECM2 alone, human follicles lost their spherical shape, and granulosa cells spread throughout the hydrogel and proliferated. It is therefore likely that oECM2 alone was not rigid enough to maintain the 3D structure of isolated human follicles, but it was not toxic and so was able to support cell attachment and proliferation. While alginate alone was superior in terms of follicle recovery rate to other hydrogels combinations, it is unfortunately unsuitable alone for possible future clinical application in the TEO due to its low degradability and poor revascularization after transplantation.

However, given that no differences were observed between human ovarian follicle viability and growth in alginate and oECM2-alginate combinations, the addition of alginate to oECM2 may well improve scaffold rigidity. The hypothesis that scaffold rigidity may influence follicle survival has already been posited. Hornick et al. showed that when isolated nonhuman primate preantral follicles were cultured in 0.5% (soft) and 2% (stiff) alginate, follicles maintained their spherical structure and survived only in the stiffer alginate (2%). Additionally, Lambracht et al. characterized the chemical and physical properties of different hydrogels and demonstrated that SLM 100 alginate (1%) exhibited rigidity (~4 KPa) similar to bovine and human ovarian tissue.

Although no other studies to date have reported on ovarian ECM-derived hydrogels, Pors et al. recently obtained encouraging findings with isolated human ovarian follicles cultured in a decellu-

larized human ovarian ECM scaffold. Unlike our study, human ovarian follicles were first wrapped between two sheets of previously cultured ovarian cells and only then placed together inside the decellularized ovarian scaffold. Hence, the presence of ovarian stromal cells, essential for appropriate bidirectional communication with follicles, as well as secreted autologous ovarian cell-derived ECM, may have contributed to follicle survival. Therefore, in order to support longer human follicle survival in vitro, the addition of ovarian cells should be considered in future studies.

We are aware that this is a pilot study, and it has therefore sev-

eral limitations, such as the lack of some critical functional assays and longer periods of in vitro culture. Moreover, further in vivo investiga-
tions (e.g., xenografting model) are required to confirm the hydrogel potential for supporting human folliculogenesis and assessing its degrada-
tion and vascularization. Finally, some tests with immunocompe-
tent animal models should be performed to evaluate the host immunoresponse to the hydrogel.

It is equally important to point out that since this is a pilot study, we decided to use ovaries from only two cows. However, ovaries vary among animals, considering several aspects, such as the estrous cycle, animal age, breed, and health status. Hence, to decrease the hydrogel composition variability, the best option is to pool ovaries from several animals. This is also necessary because a single ovary would be insufficient to produce enough ECM-derived hydrogel.

5 | CONCLUSIONS

Both hydrogels were able to support mouse follicle survival in vitro. However, taking into account that requirements to support follicle survival in TEO are species-specific, we selected oECM2 as it proved better than oECM1 to culture human ovarian follicles. Nevertheless, pure oECM2 was not able to sustain human follicle survival in vitro, probably due to its relative softness compared to human ovarian tis-

sue. On the other hand, when alginate was combined with oECM2, up to 65% of fully viable and growing follicles were detected on day 7 of culture. Since alginate alone is not suitable for TEO clinical translation, combined oECM2-alginate hydrogels may offer an innovative strat-
egy. We hope that this derived oECM hydrogel may function as a
biomimetic environment, with retention of ovarian ECM proteins essential for proper follicle development, greater degradability, and enhanced revascularization after grafting.

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CONFLICT OF INTEREST
None of the authors have any competing interests to declare in relation to the topic.

AUTHOR CONTRIBUTIONS
MCC, JV, LJW, CAA were responsible for the study design and manuscript preparation. MCC, JV, EO, NT, AV performed the experimental procedures and acquired the data. AdR, LJW, CAA and MMD assisted in revising the manuscript and added critical feedback. All the authors are aware of this submission. LJW and CAA contributed equally to this manuscript and should be considered joint senior authors.

DATA AVAILABILITY STATEMENT
All data generated or analysed during this study are included in this article and are available with the corresponding author upon reasonable request.

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REFERENCES


57. Mortiaux L. Physical aspect of the artificial ovary: characterization of human ovarian tissue and fibrin scaffolds. École polytechnique de Louvain, Louvain-la-Neuve, Belgium: Université Catholique de Louvain; 2016.


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