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Research Article

Culture of Immature Ovarian Follicles within Decellularized Ovary Enhances Oocyte Maturation and Improves In vitro Fertilization Results

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Abstract

The goal of this study is to improve methodologies that define the maturation of ovarian follicles and enhance in vitro fertilization by employing decellularized ovaries. Preantral follicles of mice were cultured for 14 days in both the decellularized ovary and two- dimensional (2D) conditions. The oocyte maturation rate, fertilization rate, and the subsequent embryo development rate were assessed in 2D and the decellularized ovary and compared to in vivo condition. Additionally, the gene expression profile of IGF1R, integrin $\alpha v\beta 3$, Cox2, Caspase-3, Bax, and Bcl2 l1 was determined in blastocysts. The culture in the decellularized ovary showed a significantly higher number of MII oocytes in comparison to the 2D culture ($P < 0.05$). Compared to in vivo, both the 2D and the decellularized ovary cultures exhibited significantly lower percentages of MII oocytes, 2PN, two-cell, cleavage, and blastocyst ($P < 0.05$). In the decellularized ovary culture, significantly higher percentages of 2PN and blastocyst were observed ($P < 0.05$) compared to the 2D culture. The gene expression level of *IGF1R* and *Cox2* in blastocysts from both the 2D and the decellularized ovary cultures was markedly lower compared to in vivo. However, the gene expression levels of Integrin αv and $\beta 3$ were comparable in blastocysts derived from in vivo and decellularized ovary-matured oocytes. Blastocysts derived from decellularized ovary-matured oocytes showed a higher bcl211 expression level compared to the blastocysts from 2D ($P < 0.05$). Employing decellularized ovarian tissues methodologies for in vitro maturation of oocytes provides a promising avenue towards generating embryos with improved implantation potential.

Keywords: extracellular matrix, oocytes, embryo development, tissue engineering, ovary

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1. Introduction

Ovarian tissue engineering promises to restore fertility in young women with malignant disorders, who are undergoing gonadotoxic treatments [1]. Ovarian tissue cryopreservation prior to treatment, and culture of immature follicles following treatment, are potential approaches to preserving and restoring fertility in cancer survivors [2]. In particular, in vitro maturation (IVM) of oocytes aims to preserve fertility and aid in the management of ovarian hyperstimulation syndrome [3]. However, while IVM has advantages, the chances of conceiving using IVM gametes are disproportionately low compared to using oocytes that have matured in the ovaries [4].

Folliculogenesis, a complex process involving interactions among various cell types such as granulosa cells, and oocytes, depends on the extracellular matrix (ECM) providing the necessary structural support throughout the growth and maturation process of follicles [5]. The ECM also regulates cell communication, differentiation, and steroid production [6]. Within the ovaries, follicles are exposed to a three-dimensional structure surrounded by supportive cells and ECM. Therefore, replicating this 3D niche in vitro provides a more accurate representation of the conditions essential for proper follicular growth and maturation [7]. Furthermore, the 3D structure of ovarian ECM serves as a reservoir for specific signaling molecules (paracrine regulatory factors) that are crucial to the progression of folliculogenesis [8]. Studies have demonstrated that 3D culture systems more accurately replicate the physiological conditions of ovaries than traditional 2D cultures, offering a platform that better mimics the complexity of the ovarian microenvironment [9-11]. The aim of employing 3D culture for ovarian follicles is to improve oocyte quality and maturity, ultimately enhancing the success rate of fertilization [11]. Decellularized tissues serve as scaffolds for tissue engineering and regenerative medicine, providing a supportive environment for tissue regeneration and reducing the risk of rejection when repopulated with the patient cells [12-14]. Previous studies, including our own, have demonstrated the efficacy and beneficial effects of ovarian decellularization [14-16] in maturing immature follicles [17]. Early embryo development requires the synchronized influence of steroid hormones, growth factors, and integrins. The insulin-like growth factor 1 receptor (*IGF1R*) is vital for embryonic development, influencing cell growth, differentiation, and survival [18, 19]. The $\alpha v\beta 3$ integrin, a cell adhesion molecule, plays a noteworthy role in blastocyst development, and in the critical stage of embryogenesis, facilitating successful attachment and implantation of the blastocyst into the uterine cavity [20]. Cyclooxygenase (Cox2), orchestrating the synthesis of prostaglandins, is involved in blastocyst implantation by regulating uterine receptivity, uterine contractions, immune responses, promoting trophoblast invasion, and contributing to the overall success of pregnancy [21].

In the current study, we used both 2D and decellularized ovaries culture systems for cultivating immature follicles, successfully yielding mature oocytes. Subsequently, mature oocytes underwent in vitro fertilization (IVF), and the progression towards blastocyst formation was examined. Gene expression levels of *IGF1R*, integrin $\alpha v\beta 3$, Cox2, Caspase-3, Bax, and Bcl2l1 were assessed in embryos derived from

mature oocytes. Finally, rates of maturation, fertilization, and blastocysts gene expression were assessed in embryos generated from both 2D and decellularized ovary cultures and compared to those generated in vivo.

2. Materials and Methods

2.1. The experimental design

As illustrated in the graphical abstract, the study involved two phases. In Phase 1, ovaries from mature Wistar rats were collected and subjected to decellularization. Verification of decellularization was accomplished through histological examination, DNA content analysis, and scanning electron microscopy. In Phase 2, preantral follicles from immature mice were harvested and cultured for 12 days using 2D and decellularized rat ovaries, followed by the addition of hCG to induce oocyte maturation. Subsequently, mature oocytes from both 2D and decellularized rat ovaries underwent IVF, and the inseminated oocytes were cultured. Various parameters, including fertilization rate, cleavage, and blastocyst formation, as well as the gene expression profile of IGF1R, integrin $\alpha v\beta 3$, Cox2, Caspase-3, Bax, and Bcl2 l1, were evaluated in blastocysts. Additionally, to compare these parameters with those of oocytes matured in vivo, adult female mice were superovulated, and mature oocytes were harvested and utilized for IVF. The phases of the study are depicted in Figure **1**.

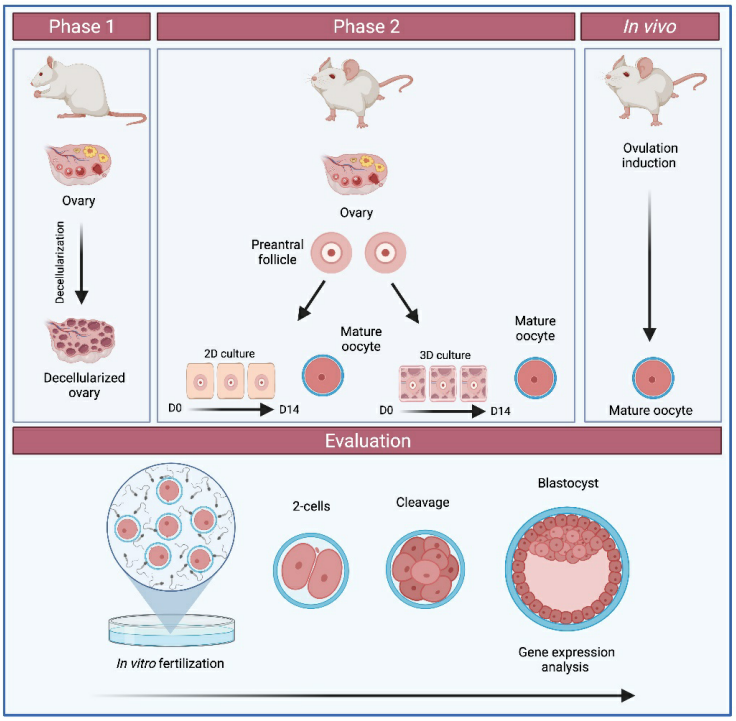

Figure 1: The three phases of the study.

2.2. Animals

28 mature female Wistar rats (weighing 200 ± 20 g) were obtained from the animal facility at Shiraz University of Medical Sciences, Iran. The animals were housed in standard polyester cages under controlled conditions of temperature, humidity, and a 12-hour light/dark cycle. The estrus phase of rats was determined through daily vaginal smear testing, and euthanasia was performed using a CO2 chamber. Subsequently, both ovaries were harvested and used for decellularization. An ethical certificate was acquired from the Animal Ethical Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.1020) for all animal procedures.

2.3. Decellularization of the ovarian tissue

The process of decellularization of ovaries followed the methodology detailed in our previous study, using sodium lauryl ester sulfate [17]. The decellularization and successful cell removal were confirmed by histochemical staining including hematoxylin and eosin, Hoechst, Masson's trichrome, and alcian blue stains, as well as DNA content analysis and scanning electron microscopy in our previous study.

2.4. Analysis of decellularization

2.4.1. Histological analysis

The effectiveness of cell removal was assessed using staining with hematoxylin and eosin (H&E) as well as Hoechst stains. These sections were then examined using both light microscopy (Olympus BX61, Tokyo, Japan) and fluorescence microscopy (Olympus BX51, Japan), each equipped with a digital camera (Olympus DP73).

2.4.2. Extracellular matrix analysis

Preservation of collagen post-decellularization was evaluated by staining both decellularized and intact ovaries with Masson's trichrome stain (Bancroft and Gamble 2008). To detect glycosaminoglycan (GAG) content in the decellularized ovary, slides were stained with 1% Alcian blue (Sigma-Aldrich) at pH 2, followed by counterstaining with 0.1% Nuclear Fast Red in 5% aluminum sulfate for 10 minutes (Bancroft and Gamble 2008).

2.4.3. DNA content assay

To verify the successful extraction of cells from ovarian tissues during the decellularization process, the DNA content of both intact and decellularized ovaries (n = 3 per group) was evaluated using a

QIAamp® DNeasy Blood and Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The DNA yield (ng/μL) of each sample was measured spectrophotometrically in triplicate based on the optical density at γ = 260 nm, using the NanoDrop® ND-1000 instrument (NanoDrop Technologies Inc., Wilmington, DE, USA).

2.4.4. Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to assess the microarchitecture of the ovarian tissue post-decellularization. Three decellularized ovaries were fixed following the protocol outlined by Kargar-Abarghouei et al. (2018). Subsequently, the samples were coated with a fine layer of gold using a Q150R ES sputter coater (Quorum Technologies, London, UK) and visualized using a VEGA3 microscope (TESCAN, Czech Republic).

2.4.5. MTT assay

To evaluate cytocompatibility, the decellularized lyophilized ovaries underwent UV sterilization, and human Wharton's jelly mesenchymal stem cells were seeded onto each at a density of 1.0 × 106 cells/decellularized ovary and cultured at 5% CO2 and 37˚C. Subsequently, they were immersed in DMEM/F12 medium (Shell Max; A2930) containing 10% fetal bovine serum (FBS, Gibco, Paisley UK), 2 mM L-glutamine (Bioidea, Tehran, Iran), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) for 2 hours under the same conditions. An equivalent number of Mesenchymal stem cells was seeded in a 2D conventional condition as the control culture. Cellular viability was assessed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay (M5655; Sigma-Aldrich) after 1, 3, and 7 days of culturing. Following addition of the MTT solution (1 mg/mL of MTT in DMEM), the cultures were incubated for 3 hours. Formazan crystals were dissolved with 300 μL of dimethyl sulfoxide (Sigma-Aldrich) for 15 minutes, and the optical density (OD) was measured at a wavelength of 595 nm.

2.5. In vitro studies

2.5.1. Isolation of preantral follicles

After anesthesia using CO2, the ovaries of 14-day-old mice (n=28) were removed, and under the precise observation of a stereomicroscope and in handling medium (GMOPSTM, Vitrolife, Goteborg, Sweden), dissection was conducted. The preantral follicles were carefully collected using a flamed/pulled Pasteur pipette (Normax, Portugal). The harvested follicles were then characterized by centrally located oocytes and the presence of two to three compact layers of granulosa cells, each with an average diameter ranging from 110 to 130 µm. Preantral follicles were randomly subjected to both 2D and decellularized

ovary culture conditions. This controlled division aimed to explore and compare the effects of different culture environments on the development and maturation of preantral follicles.

2.5.2. In vitro culture medium

The preantral follicles underwent a cultivation process within either 2D or decellularized ovary systems, utilizing a carefully formulated medium. This medium contained α-Minimal Essential Medium (α MEM, Shellmax Co., China), providing a well-balanced and nutrient-rich foundation for the culture of follicles. To enhance the follicular development and maturation process, the medium was supplemented with essential components, including recombinant follicular stimulating hormone (FSH), fetal bovine serum (FBS), insulin-transferrin-selenium (ITS), and penicillin/streptomycin.

2.5.3. Two-dimensional culture

In each replicate, 10 preantral follicles (Total number=280) were placed in 50 µL of the abovementioned culture medium, and pre-incubated for 24 hours at 37°C. This medium was covered with mineral oil to create a controlled and stable environment. The entire culture process took place in a humidified incubator set at a temperature of 37˚C, with a balanced concentration of 5% CO2. This controlled environment provided optimal conditions for the follicles to undergo development and maturation over 14 days. To ensure the continuous provision of essential nutrients and maintain a conducive environment, the medium was replaced with a fresh one every other day.

2.5.4. Three-dimensional culture in decellularized ovary

The process involved the infusion of decellularized ovaries with a culture medium for 5 minutes. Subsequently, a cohort of 10 preantral follicles was embedded in each decellularized ovary (total number = 280). Following this, the decellularized ovaries with the embedded follicles were placed within a 96-well culture dish and exposed to the same environmental conditions as those in the 2D culture. To monitor and track potential emerging follicles, examination was conducted during the regular medium changes. The discarded medium underwent close, careful observation of follicles that might have exited from the decellularized ovary.

2.5.5. Oocyte maturation determination

On the 13th day of the experiment, human chorionic gonadotropin (hCG) was introduced into both the 2D and decellularized ovary culture systems. The addition of hCG was a deliberate step aimed at triggering

the maturation of oocytes within the experimental setup. Twenty-two hours post-hCG administration, the decellularized ovaries were meticulously dissected, and the cumulus-oocyte complexes were carefully collected from the decellularized ovaries. Subsequently, 1% hyaluronidase was used to separate the cumulus cells around the oocyte and an inverted microscope was employed to assess the maturation status of the oocytes. This step involved a thorough examination to determine the degree of maturation achieved by the oocytes in both the 2D and decellularized ovary culture systems. The examination focused on categorizing the oocytes based on their maturation stages, including the germinal vesicle (GV), metaphase I (MI), metaphase II (MII), or by identifying any that showed signs of degeneration.

2.6. In vivo studies

2.6.1. Harvesting in vivo matured oocytes

To compare the maturation rate, fertilization rate, and developmental potential of mature oocytes obtained from the 2D and decellularized ovaries to those matured in vivo, adult female Balb/c mice (n=28) were superovulated by intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin (GonaserVR, HIPRA, Amer, Spain), followed by injection of 10 IU human chorionic gonadotropin (hCG) (LG Life Sciences, Seoul, South Korea) 48 hours later. Fourteen hours post-hCG injection, the oviducts were transferred into a handling medium (GMOPSTM, Vitrolife, G€oteborg, Sweden) which was pre-incubated for 24 hours at 37°C. The oviducts were dissected with two insulin syringes under a stereomicroscope (Nikon, Tokyo, Japan), and the released oocyte cumulus complexes were collected by flamed/pulled Pasteur pipettes and placed in drops of 1% hyaluronidase (Vitrolife) for separation of cumulus cells. The percentage of GV, MI, MII, and degenerated oocytes was determined, and mature oocytes were harvested and used for IVF.

2.7. In vitro fertilization

2.7.1. Sperm preparation

Mature Balb/c mice were euthanized, and their epididymides were removed and chopped into smaller pieces. Subsequently, the epididymal tissues were placed in a G-IVF medium (Vitrolife, Gothenburg, Sweden), and incubated for an hour at 37°C and 5% CO2 to obtain capacitated spermatozoa. After incubation, the sperm count was accurately determined using a Neubauer cell counting chamber, following WHO guidelines from 2010.

2.7.2. In vitro fertilization

Mature oocytes from 2D, decellularized ovary, and in vivo culture systems were subjected to in vitro insemination with 1×106 spermatozoa/mL in GIVF medium for 5 hours at 37°C and 5% CO2. Then

inseminated oocytes were collected, washed, and assessed for fertilization rate. Subsequently, fertilized oocytes were transferred to GTL medium (Vitrolife, Gothenburg, Sweden), and the developmental progress of embryos was tracked. The number of two-cells, cleavage, and blastocysts were counted at 24, 48, and 96 hours using an inverted microscope. This approach provided comprehensive insights into early embryonic development, including the rate of fertilization, cleavage, and blastocyst formation.

2.8. Gene expression levels of IGF1R, Integrin v3, Cox2, Caspase-3, Bax, Bcl2l1

Real-time polymerase chain reaction (RT-PCR) was employed to assess the expression levels of IGF1R, integrin $\alpha v\beta 3$, Cox2, Caspase-3, Bax, and Bcl2l1 in embryos. Total RNAs were extracted from 3 pools (n = 3) each containing 25 blastocyst embryos using an RNA extraction kit (CinnaGen Co., Iran). The quantity and quality of RNA were determined by the optical density ratio at 260/280 nm using NanoDropTM (Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis (1%). The concentration of total RNA was adjusted to 1000 ng/mL. The first-strand complementary DNA (cDNA) was synthesized using the RevertAid^{TM} first-strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). qRT-PCR was performed on Applied Biosystems Step-One using Real Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark). Primers were designed based on mouse DNA sequences obtained from the Genebank Primer-BLAST Online Program (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) served as a reference gene. Amplification signals from different samples were normalized to the Gapdh cycle threshold (Ct), and the delta-delta CT (2-ΔΔCT) method was applied to compare mRNA levels between 2D and decellularized ovary cultures, representing a fold change in data analysis. All RT-PCR experiments were conducted in triplicate for accuracy and reliability.

Table 1: The sequence of primers was used for quantitative real-time RT-PCR.

Gene	Nucleotide sequences (5′–3′)	Fragment size (bp)
IGF1R	F: TCCTGTGTTCTTCTATGTCC R: CCTGCTGTTATTTCTCTTTCTAT	145
Integrin αv	F: TCAAGGAGGATTCAGCATT R: CCACAGAGTAACCCAAATAAC	215
Integrin β3	F: CCTTGCTACTCTGCTCATCTG R: GCTCTGGCTCGTTCTTCC	154
Cox2	F: TGCCTGGTCTGATGATGTATGC R: TGAGTATGAGTCTGCTGGTTTGGA	126
Bcl2l1	F: GCAGGTATTGGTGAGTCGGA R: CTCGGCTGCTGCATTGTTC	130
Bax	F: TGGAGATGAACTGGACAGCAAT R: TAGCAAAGTAGAAGAGGGCAACC	155
Caspase-3	F: AGGGGTCATTTATGGGACA R: TACACGGGATCTGTTTCTTTG	115
Gapdh	F: TGTTTCCTCGTCCCGTAGA R: ATCTCCACTTTGCCACTGC	106

2.9. Statistical analysis

Statistical analyses in the study were utilized using IBM® SPSS® Statistics 24.0 for Windows, presenting data as means with the standard error of the mean for clarity. One-way ANOVA and post hoc Tukey test were employed to compare the percentages of GV, MI, MII, degenerated oocytes, 2PN, 2Cell embryo, cleavage, and blastocysts among in vivo, 2D, and decellularized ovary matured oocytes. A significance level of $P < 0.05$ was applied for statistical significance.

3. Results

The histochemical analysis revealed that the ovarian tissue maintained its structural integrity after undergoing decellularization. Alcian blue staining indicated that there was still some retention of GAG content following the decellularization process. Masson's trichrome staining highlighted the presence of intact collagen fibers in the decellularized ovaries, exhibiting a distribution pattern similar to that of normal tissue. Moreover, both H&E and Hoechst staining demonstrated the complete absence of cells within the decellularized ovarian samples. Overall, the results of the histochemical staining suggest that the decellularization procedure effectively preserved the majority of the ECM while successfully eliminating cellular components (Figure **2**).

Upon examination through scanning electron microscopy, it became evident that the microarchitecture of the decellularized ovary had been carefully preserved. Within the tissue, a diverse array of pores of various sizes was observable, occupying spaces that were previously filled by follicles, interstitial cells, and vessels. Notably, the SEM image provided a detailed glimpse of the intricate network of collagen fibers, indicating that this essential structural component remained intact within the decellularized ovary (Figure **2**).

A cut section of an intact ovary showed spaces filled with cells. In the decellularized ovary, the overall architecture of the ovarian extracellular matrix remained intact, with visible spaces previously occupied by growing follicles, blood vessels, and stromal cells. SEM images of the decellularized ovary's surface confirmed the successful decellularization process.

Microscopic images of paraffin-embedded sections of adult rat ovarian tissues were examined using H&E and Hoechst stains, Masson's trichrome, and Alcian blue to assess cell removal and preservation of collagen fibers and glycosaminoglycans before and after decellularization. H&E and Hoechst staining demonstrated significant cell removal from the ovarian tissue as a result of the decellularization process. Masson's trichrome and Alcian blue staining confirmed the preservation of collagen and glycosaminoglycans, respectively, following decellularization.

The quantitative DNA analysis revealed a significant decrease in the DNA content of the decellularized ovaries compared to the intact ovaries ($P < 0.001$), indicating successful cell removal (Figure **3**.A).

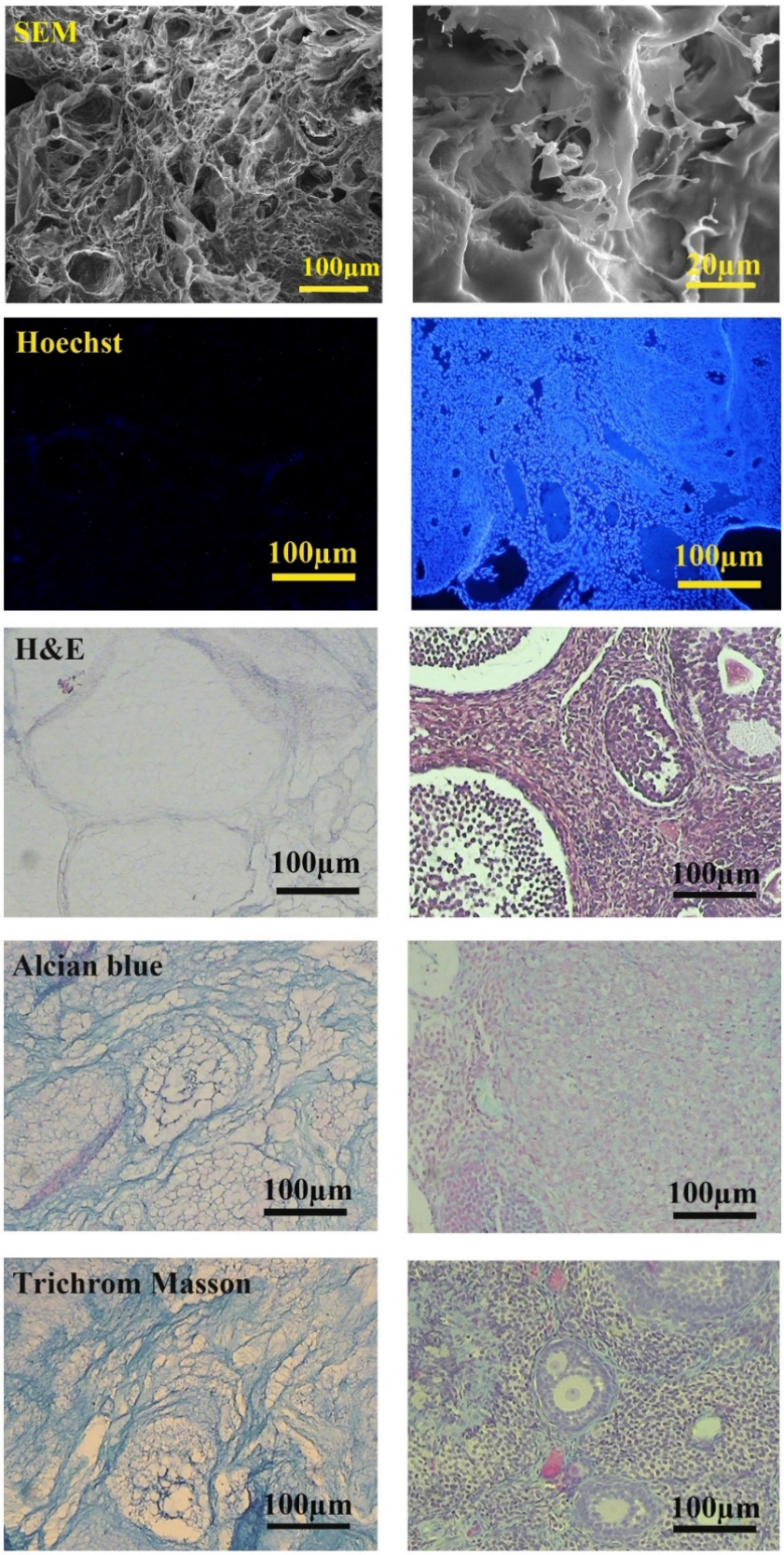

Figure 2: Scanning electron microscopy and histological analysis of the intact and decellularized ovaries.

An evaluation of the cytocompatibility of the decellularized ovary was conducted by assessing the viability and proliferation of seeded human Wharton's jelly mesenchymal stem cells. The MTT assay confirmed the viability of human Wharton's jelly mesenchymal stem cells on the decellularized ovaries, and the comparison of optical density (OD) values at various time points indicated proper cell proliferation within the decellularized ovaries. In all early stages of culture, cell proliferation on the decellularized ovaries was similar to that in the 2D culture ($P > 0.05$) (Figure **3**.B).

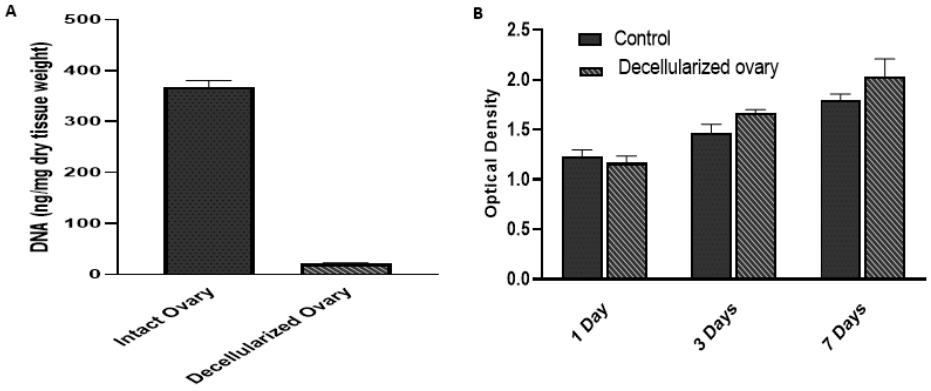

Figure 3: A: DNA content of intact and decellularized ovary. The DNA content in decellularized ovarian tissues showed a substantial reduction compared to intact ovarian tissues (n = 3), with a statistically significant difference ($P < 0.001$). B: An evaluation of the cytocompatibility of the decellularized ovary after seeding of human Wharton's jelly mesenchymal stem cells and culturing for 1, 3, and 7 days in comparison to culture media. The MTT assay revealed that cell proliferation on the decellularized ovary is similar to the control culture up to day 7 ($P > 0.05$). Data are presented as the mean ± standard error of the mean (SEM).

As indicated in Table 2, notable differences were observed among the in vivo, 2D, and decellularized ovary culture groups concerning oocyte maturation. The percentage of MII oocytes produced in both the 2D and decellularized ovary culture groups was significantly lower compared to the in vivo group ($P < 0.001$ and $P = 0.036$ respectively). Remarkably, the decellularized ovary culture group showed a significantly higher percentage of MII oocytes in comparison to the 2D group ($P < 0.001$). Nonetheless, the percentage of GV and MI oocytes were significantly lower in the decellularized ovary culture compared to the 2D culture ($P = 0.003$ and $P = 0.012$, respectively).

In the 2D culture group, a significantly higher percentage of GV, MI, and degenerated oocytes was found compared to the in vivo group ($P < 0.001$, $P < 0.001$, and $P < 0.001$respectively). Interestingly, no significant differences in the mentioned parameters between decellularized ovary and in vivo conditions were found ($P > 0.05$).

In comparison to in vivo, the percentage of 2PN, two-cell, cleavage, and blastocyst was significantly lower in both 2D and decellularized ovary groups ($P < 0.05$). The percentage of 2PN and blastocyst was significantly higher in the decellularized ovary group compared to the 2D groups ($P < 0.001$ and $P = 0.022$, respectively).

Table 2: The percentage of GV, MI, MII, and degenerated oocytes after in vivo maturation and culture in 2D and decellularized ovary conditions.

Groups	Number of follicles	GV Oocytes (% ± SEM)	MI Oocytes (% ± SEM)	MII Oocytes (% ± SEM)	Degenerated oocytes (% ± SEM)
In vivo	501	52 (10.37±1.67) ‡	63 (12.57±1.12) ‡	368 (73.45±1.08) *	18 (3.59±0.72) ‡
2D	280	49 (17.5±1.21) #	62 (22.14±1.48) #	141 (50.35±2.49) #	28 (10±1.54)
3D	249	28 (11.24±1.19)	40 (16.06±1.65)	164 (65.86±2.12)	17(6.82±1.15)

GV: germinal vesicle, MI: Meiosis I, MII: mature oocytes
Significant difference from 2D and 3D group (P<0.05)
Significant difference from 3D group (P<0.05)
‡ Significant difference from 2D group (P<0.05)

Moreover, the decellularized ovary group exhibited higher percentages of two-cell and cleavage embryos, compared to the 2D group, although the difference was not statistically significant (P > 0.05) as outlined in Table 3.

Table 3: The percentage of oocytes matured in three groups for 2PN, two-cell, cleavage, and blastocyst.

Groups	Number of MII Oocytes	2PN (% ± SEM)	Two-Cells (% ± SEM)	Cleavage (% ± SEM)	Blastocyst (% ± SEM)
In vivo	368	336 (91.3±1.45) *	285 (77.44±1.36) *	253 (68.75±1.55) *	224 (60.86±1.99) *
2D	141	98 (69.5±2.32) #	82 (58.15±2.86)	67 (47.51±3.26)	47 (33.33±3.09) #
3D	164	134 (81.7±2.79)	107 (65.24±2.22)	89 (54.26±2.51)	73 (44.51±2.45)

Significant difference from to 2D and 3D groups (P<0.05)
#Significant difference from 3D group (P<0.05)

The mRNA levels of three critical genes involved in blastocyst development and implantation (IGF1R, integrin $\alpha v\beta 3$, and Cox2) were assessed in harvested blastocysts from different groups of mice using qRT-PCR. The results revealed that IGF1R mRNA levels were significantly higher in blastocysts from in vivo-matured oocytes compared to both the 2D and decellularized ovary groups (P = 0.006 and P = 0.026, respectively). However, there was no significant difference in IGF1R levels between blastocysts from the 2D and decellularized ovary -matured oocytes (P > 0.05). Integrin αv and $\beta 3$ levels were significantly elevated in blastocysts from in vivo-matured oocytes compared to 2D (P = 0.007 and P = 0.004, respectively). Additionally, the expression of αv and $\beta 3$ subunits of integrin mRNA was higher in decellularized ovary culture blastocysts compared to 2D culture (P = 0.036 and P = 0.004, respectively). Nevertheless, there was no significant change in αv and $\beta 3$ levels between blastocysts from in vivo-matured and decellularized ovary-matured oocytes (P > 0.05). The results also demonstrated a marked downregulation in Cox2 gene expression in blastocysts from both 2D and decellularized ovary -matured oocytes compared to those from in vivo-matured oocytes (P < 0.001 and P < 0.001, respectively). However, Cox2 expression was statistically comparable in blastocysts from 2D and decellularized ovary -matured oocytes (P > 0.05)(Fig. 4). As indicated in Fig. 5, the Caspase-3 expression level was greater in blastocysts obtained from in vivo-matured oocytes compared to those matured in 2D and decellularized ovary conditions, although the difference was not statistically significant (P > 0.05). The Bax mRNA gene expression level was

comparable between blastocysts from in vivo-matured and decellularized ovary -matured oocytes (P > 0.05). However, it was higher in blastocysts from 2D-matured oocytes when compared to those from in vivo-matured oocytes (P = 0.048). There was no significant difference in its expression between 2D and decellularized ovary groups (P > 0.05). The Bcl2l1 mRNA level in harvested blastocysts from in vivo-matured, 2D-matured, and decellularized ovary-matured oocytes was similar (P < 0.05). Nevertheless, its expression was elevated in blastocysts from decellularized ovary-matured oocytes compared to those matured in 2D (P=0.03).

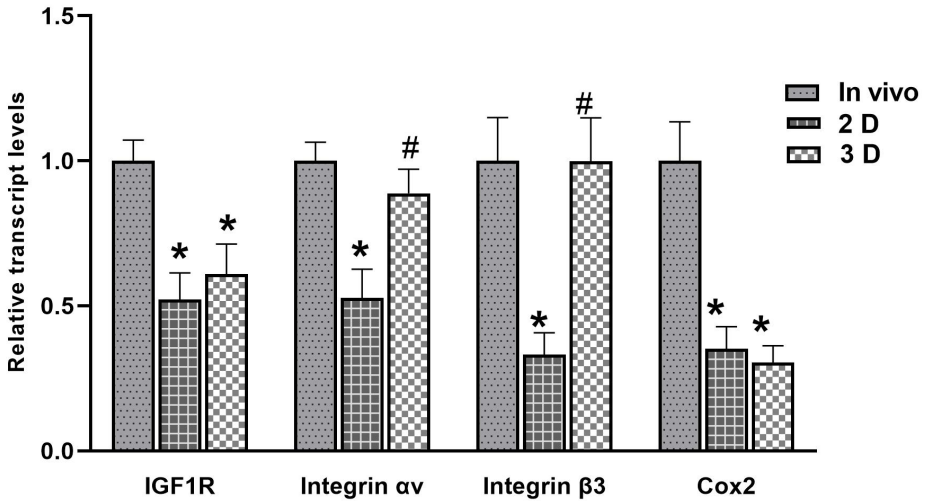

Figure 4: The gene expression levels of IGF1R, Integrin αv, Integrin β3 and, Cox2 were analyzed in the resultant blastocysts. *Indicates a significant difference from the in vivo group (P>0.05). # Shows a significant difference from the 2D group (P>0.05).

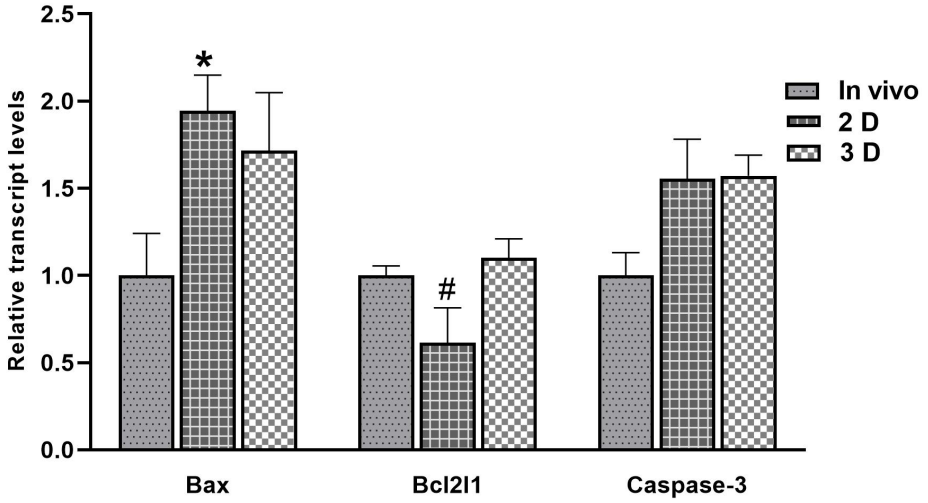

Figure 5: The gene expression levels of apoptotic-related genes in resultant blastocysts. * Indicates a significant difference from the in vivo group (P > 0.05). # Shows a significant difference from the 2D group (P > 0.05).

4. Discussion

Building upon our previous study [17], which demonstrated the efficacy of the decellularized ovary in maturing immature follicles and producing mature oocytes, we aimed to assess the fertilization potential of mature oocytes harvested from decellularized ovaries. Additionally, we assessed the rate of fertilization and blastocyst formation, as well as determined the gene expression levels of IGF1R, integrin $\alpha v\beta 3$, Cox2, Caspase-3, Bax, and Bcl2l1 in the blastocysts.

To evaluate follicle functionality in both 2D and decellularized ovary conditions, oocyte maturation was induced. We observed that the decellularized ovary significantly enhanced oocyte maturation, and reduced the percentage of GV, MI, and oocytes compared to the conventional 2D culture system. This indicated an accelerated rate of oocyte maturation within the three-dimensional environment of ovarian ECM, emphasizing its benefits for oocyte development. Although the oocyte maturation in the 2D culture group did not reach the level of in vivo conditions, the oocyte maturation in the decellularized ovary culture group did reach a level comparable to in vivo conditions. The rates of 2PN, two-cell, cleavage, and blastocyst formation were significantly higher in oocytes matured in vivo compared to those oocytes matured in 2D and decellularized ovary conditions. Additionally, significantly higher percentages of blastocysts were obtained from oocytes matured in the decellularized ovary than the 2D culture system, indicating that the decellularized ovary culture system not only improves fertilization but also promotes subsequent embryonic development. Francés-Herrero et al. used hydrogels derived from decellularized bovine ovaries for the culture of mouse secondary follicles [22]. In their study, follicles grown on the hydrogel coating exhibited similar cumulus-oocyte complex recovery rates compared to standard conditions. Furthermore, oocytes in hydrogel displayed normal meiotic spindle formation and homogeneous mitochondrial distribution within their ooplasm. Moreover, a higher proportion of oocytes cultured on the hydrogel coating reached the 4-cell embryo stage compared to those cultured in standard media [22]. Their results are in line with the results of the current study.

Pennarossa et al. conducted a decellularization protocol on entire porcine ovaries [23]. They repopulated the obtained bioscaffolds with either porcine ovarian cells or epigenetically erased porcine and human dermal fibroblasts. The decellularized ECM-based scaffold was identified as a conducive niche for the ex vivo cultivation of ovarian cells, which effectively directed the differentiation, fate, and viability of the epigenetically reset cells [23]. In another study conducted by Nikniaz et al., a different decellularization method was used for bovine and human ovaries, followed by the culture of mice with immature preantral follicles within the decellularized ovary. The results indicated that a substantial population of the follicles transferred into the decellularized scaffold remained viable after 7 days of cultivation [24]. In another study, primary ovarian cells of mice were encapsulated within freshly decellularizing ovaries derived from female swine and then implanted in ovariectomized mice. The results demonstrated significant signals in neoangiogenesis, cell proliferation and survival, sex hormone secretion, and expression of

primary ovarian cell markers [25]. Park et al. utilized hydrogels from the decellularized porcine ovary for culturing the preantral follicles of mice and comparing them with other biomaterials such as collagen and matrigel. In this study, in vitro maturation was induced and the maturation rate and oocyte quality were evaluated as well. The MII oocytes derived from antral follicles were further assessed for embryonic development following parthenogenetic activation. The preantral follicles cultured in a biomimetic ovary with a decellularized porcine scaffold showed more enhanced formation of antral follicles and mature oocytes than those cultured in other biomaterials. The percentage of MII oocytes retrieved from the ECM hydrogel was significantly higher than that of collagen. MII oocytes with normal spindle organization and chromosomal arrangement were observed. Mature oocytes retrieved from the ECM hydrogel developed into two-cell embryos after parthenogenetic activation [26]. Chitti et al. showed that the decellularized bovine ovary can support the follicle's growth and viability in mice and human ovaries [11].

All these studies are inconsistent with the results of our study. Our assessments went beyond evaluating the influence of ovarian ECM on oocyte maturation and its consequent progression. We also explored the expression levels of critical factors in the developmental processes of the preimplantation embryo. The convergence of IGF1R, integrin $\alpha v\beta 3$, and Cox2 signaling pathways is evident, playing an essential role in various stages of embryo development [20]. For instance, during angiogenesis, integrin-mediated adhesion and IGF1R-driven cell survival are intricately linked, and Cox2 can modulate these processes by impacting vascular development [18]. The coordination of IGF1R, integrin $\alpha v\beta 3$, and Cox2 signaling is crucial for the orchestration of embryo development, regulating fundamental processes such as cell adhesion, proliferation, survival, and vascularization [21].

In our study, we observed that the expression levels of the IGF1R and Cox2 genes in blastocysts derived from oocytes matured in both 2D and decellularized ovary environments were significantly lower compared to those from in vivo matured oocytes. However, the gene expression levels of both subunits of integrin $\alpha v\beta 3$ in blastocysts derived from oocytes matured in decellularized ovary environments were similar to those from in vivo-matured oocytes. Therefore, while the diminished expression of IGF1R and Cox2 genes in blastocysts from decellularized ovaries raises concerns about their implantation potential, the comparable gene expression levels of integrin $\alpha v\beta 3$ subunits suggest a certain level of competency in the decellularized ovary for producing blastocysts with implantation capabilities similar to those originating in vivo. Further investigations are warranted to fully elucidate the molecular mechanisms underlying these observations and to optimize conditions for improving implantation potential in embryos derived from decellularized ovaries.

Apoptosis is a finely tuned mechanism crucial for accurate embryo development and blastocyst formation, removing unwanted cells and ensuring proper cell numbers, and thus contributing to the creation of a healthy organism [27]. Therefore, securing an appropriate apoptotic balance is essential for the correct embryo development and overall success of the process. In our examination of gene expression profiles related to apoptosis in blastocysts derived from different oocyte maturation

environments, we focused on Caspase-3, Bax, and Bcl2l1. While Caspase-3, categorized as an executioner caspase, did not show statistical significance, Bax and Bcl2l1 exhibited distinct patterns, underscoring the complexity of apoptosis regulation during oocyte maturation and its potential impact on blastocyst development.

The Bcl2l1 mRNA, identified as an anti-apoptotic gene in harvested blastocysts, level of expression was similar in blastocysts derived from in vivo-matured and from decellularized ovary-matured oocytes. Furthermore, its expression was higher in decellularized ovary culture compared to 2D. In contrast, the pro-apoptotic gene Bax displayed increased expression in blastocysts from 2D-matured oocytes compared to those from in vivo and decellularized ovary-matured oocytes. Therefore, the apoptotic process in decellularized ovary culture tends to mimic the natural state more closely.

5. Conclusion

Employing decellularized ovaries contribute to creating a supportive environment crucial for follicle maturation. The results confirm the vital role of the ovarian ECM in supporting follicle growth and regulating essential factors for the successful development of follicles and oocytes. The methodology employed in the current study promises to improve the success rates of IVF procedures by showcasing the potential of using the decellularized ovary culture system to successfully generate mature oocytes.

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