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PAPER

The effect of collagen hydrogel on 3D culture of ovarian follicles

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Abstract

The in vivo function and phenotype of ovarian follicle cells are determined by many factors. When these cells are removed from the in vivo microenvironment and grown in a 2D in vitro environment, the function of the follicular cells is difficult to preserve. A collagen hydrogel was used to examine the hormone and oocyte maturation of ovary follicles in a 3D culture system. Ovarian follicles from rats were isolated and cultured in various concentration of type I collagen hydrogels ranging from 1% to 7% (weight/volume). Differences in cell survival, follicle growth and development, sex hormone production, and oocyte maturation were seen with the modifications in the collagen hydrogel density. The results show the significance of the collagen hydrogel properties on phenotype and function maintenance of the ovarian follicles in a 3D culture system.

1. Introduction

The ovarian follicle is a distinctive structure that comprises a maturing oocyte that is enclosed by supporting cells (theca and granulosa cells). Female patients undergoing cancer therapy are at risk of developing infertility due to damage to the ovaries. Maintaining the in vitro function of ovary tissue is important for the purpose of ovarian tissue banking and fertility preservation for young girls and women undergoing cancer therapies [1, 2]. In vitro follicle culture with oocyte maturation and fertilization techniques have been recently suggested as a substitute to transplantation of ovarian tissue by decreasing the risk of reintroducing cancer cells [3]. These techniques require that the preserved immature ovarian follicles be matured for the production of oocytes that can be fertilized [4–6]. Previous studies show the importance of cell–cell communication in the proliferation and maturation of ovarian follicles [7, 8]. In traditional two-dimensional (2D) culture systems, ovarian stromal cells migrate from around the oocyte and attach onto the surface of the culture vessel, thus disrupting stromal cell–oocyte interaction [9]. The ineffectiveness of oocyte development in the 2D culture systems has been the major obstacle to in vitro maturation procedures. Therefore, three-dimensional (3D) cultures that mimic the in vivo ovarian microenvironment have been developed for in vitro ovarian cell culture [10, 11]. 3D cultures facilitate maintenance of overall structure of the follicle and provide the necessary communication pathways between the oocyte and the ovarian stromal cells, thus allowing normal follicle maturation.

Recently, many researchers have concentrated on the design of 3D culture systems using hydrogel biomaterials which provide biochemical and biomechanical support for ovarian follicle cultures [12, 13]. There is a large growing body of literature documenting the profound impact of the biophysical attributes of the extracellular matrix (ECM) on cellular behaviors, including cell proliferation, differentiation, phenotypes, and function, with 3D microenvironment using hydrogel materials being identified as a cogent cue [14–16]. The dynamic nature of 3D microenvironment remains across a wide-ranging of physiological phases from development. Many studies have directed the effects of biophysical cues on cellular function extending from cell attachment and proliferation to stem cell differentiation [17, 18]. Accordingly, elucidating the relationship between biophysical cues and cell function is required to generate in vitro microenvironments that could mimic the in vivo condition more precisely [19]. Prior studies suggest that controlling the follicle maturation in the ovarian cortex might be associated with
physical pressure and tissue rigidity [20, 21]. Therefore, it is expected that biomechanical features of 3D microenvironment should play a role in regulating ovarian cell phenotypes and function.

Since increasing evidence underscores the importance of the biophysical microenvironment in determining cell fate and development, we examined the significance of the biophysical environment in the context of the ovarian follicle maturation. The effects of collagen hydrogel was investigated by controlling the concentrations of the hydrogel and examining the maintenance of function of the ovarian follicles in vitro. Primary rat ovarian follicles were incorporated in collagen hydrogels of varying the concentration ranging from 1% to 7% (weight/volume). Furthermore, we examined the viability and structural integrity of the ovarian follicle constructs as well as the function, including the hormone production and oocyte maturation. Our study is significant in that it offers an ovarian cell culture matrix that may be tuned to contain biomechanical cues most conductive to maintenance of ovarian follicle phenotype and function.

2. Materials and methods

2.1. Ovarian follicle isolation and characterization

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest School of Medicine. Three week old female Fisher 344 rats (Charles River Laboratories, Wilmington, MA, USA) were euthanized via CO2 inhalation to obtain ovarian tissues. Ovaries were carefully dissected using an aseptic technique in a laminar flow clean bench. For the preparation procedures were performed using an aseptic technique in a laminar flow clean bench. For the isolation of the ovarian follicles. Briefly, according to the manufacturer’s instructions, the collagen solution was diluted with 10 × PBS, 1 × culture medium, and 1N NaOH to prepare the neutralized collagen solutions with various concentrations of 1, 3, 5, and 7 mg ml⁻¹, respectively. The prepared collagen solutions were placed on ice prior to the incorporation of the ovarian follicles. All of the preparation procedures were performed using an aseptic technique in a laminar flow clean bench. For inducing the gelation of each collagen solution, the solutions were incubated at 37 °C for 1 h.

After the gelation, fiber morphology of the collagen hydrogels was examined by scanning electron microscopy (SEM; Model S-2260N, Hitachi Co. Ltd, Japan). The collagen hydrogels were dehydrated with serial ethanol solutions from 50% to 100%, and then air-dried. The prepared collagen hydrogels was examined by scanning electron microscopy (SEM; Model S-2260N, Hitachi Co. Ltd, Japan).

To examine the matrix elasticity, viscoelastic properties of the collagen hydrogels were measured by an HR-2 Discovery Rheometer (TA Instruments, Newcastle, DE, USA). After neutralization of collagen solutions, gelation was induced by incubation at 37 °C for 1 h. All samples were tested within 24 h of gelation. Briefly, a 12 mm flat-ended punch was pressed into the surface of the collagen hydrogels until it reached an axial force of 0.03 N. The rheometer calculated the storage modulus (G', which measures the gels ability to ‘store’ elastic energy, as well as the loss modulus (G''), which measures the amount of energy ‘lost’ in the form of heat due to viscous forces acting on the gel. The dynamic modulus of elasticity (E) can be found as an equation of:
The program gave 12 data points along the stress-sweep for both the loss modulus and storage modulus, which were averaged before being squared in the equation for elastic modulus. All experiments were carried out in triplicate.

2.3. Ovarian follicle viability and morphology

For 3D culture, the ovarian follicles were placed in each well of 48-well culture plates and mixed with 150 µl of collagen solutions with different concentrations. After 1 h in the incubation at 37 °C in 5% CO₂ atmosphere, 400 µl of culture medium was added into each well. The culture medium was replaced with fresh medium every other day. Images of ovarian follicles were taken daily using an inverted Leica DM IRB microscope with transmitted light and phase objectives (Leica, Bannockburn, IL, USA). Follicle diameters were later measured from the outer layer of theca cells using Images 1.43 U software (Scion Image, Frederick, MD, USA). An average of two perpendicular diameter measurements was used for each follicle.

The viability of the follicles in the collagen hydrogels was determined by the LIVE/DEAD Viability Assay Kit (Invitrogen). Briefly, the culture medium was aspirated and replaced with 500 µl of PBS for 5 min. After removing PBS, 300 µl of the reagent from the LIVE/DEAD Viability Assay Kit was added to each hydrogel. The LIVE/DEAD reagent was made by mixing 2.5 µl Calcein-AM, 10 µl ethidium homodimer-1 and 5 ml of PBS. The hydrogel was incubated in the reagent in the dark for 30 min and then rinsed in PBS for 5 min. The follicles were then observed under an inverted fluorescence microscope and visualized. Green fluorescence was visualized in live cells and red fluorescence in dead cells using two different filters. The number of live and dead cells was analyzed from the composite image acquired using Image-Pro plus software version 6.3.1.542. The follicles with more than 10% dead ovarian cells considered as dead.

2.4. Production of 17β-estradiol and progesterone

To evaluate the function of the ovarian follicles in the collagen hydrogels with different matrix elasticity, 17β-estradiol and progesterone were measured in conditioned medium collected during the in vitro culture. Briefly, every 2–3 d, half of the media (200 µl) was collected and replaced with fresh medium (200 µl). The levels of 17β-estradiol and progesterone (Enzo Life Sciences, Plymouth Meeting, PA, USA) in the culture media were measured using competitive enzyme immunoassay kits following the manufacturers’ instructions. The absorbance at 405 nm was measured.
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using a spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA).

2.5. Oocyte meiotic competence

Oocyte meiotic competence was assessed by maturation after 12 d of culture. Using a 30-gauge needle, antral follicles were removed from the collagen hydrogels. Oocytes were collected from antral follicles and transferred to \( \alpha \)-MEM containing 10% FBS, 5 ng \( \text{ml}^{-1} \) epidermal growth factor (EGF) and 1.5 IU \( \text{ml}^{-1} \) human chorionic gonadotropin. The oocytes were allowed to mature at \( 37^\circ \text{C} \) in 5% \( \text{CO}_2 \) for 14–16 h. Following maturation, oocytes were denuded from the surrounding cumulus cells by treating with 0.3% hyaluronidase. Oocyte state was assessed from the light microscopy images. The numbers of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) were calculated (\( n = 5 \)).

Table 1. Rheological properties of collagen hydrogels with different concentrations.

<table>
<thead>
<tr>
<th>Collagen concentration (mg ( \text{ml}^{-1} ))</th>
<th>Elastic modulus (Pa)</th>
<th>( G' ) (Pa)</th>
<th>( G'' ) (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60 ± 12</td>
<td>56 ± 13</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>457 ± 56</td>
<td>44 ± 60</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>5</td>
<td>1351 ± 137</td>
<td>1304 ± 141</td>
<td>348 ± 31</td>
</tr>
<tr>
<td>7</td>
<td>1671 ± 157</td>
<td>1635 ± 143</td>
<td>344 ± 82</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \).

Table 2. Characterization of collagen hydrogels. (A) SEM images comparing the ultrastructure of collagen hydrogels at concentrations of (a) 1 mg \( \text{ml}^{-1} \), (b) 3 mg \( \text{ml}^{-1} \), (c) 5 mg \( \text{ml}^{-1} \), and (d) 7 mg \( \text{ml}^{-1} \). (B) The relationship between hydrogel elasticity (elastic modulus) and collagen concentration (mg \( \text{ml}^{-1} \)). Data represent the mean ± S.D. (\( n = 5 \)). * \( P < 0.05 \).

3. Results and discussion

3.1. Ovarian follicle isolation and characterization

We successfully isolated ovarian follicles from rat ovarian tissues. At 6 and 14 d in culture, the ovarian cells showed a typical epithelioid cell morphology (figure 1(A)) and expressed various enzyme and hormone markers: \( 3\beta \)-HSD, FSHR, CYP19, inhibin \( \alpha \), LH and estradiol (figures 1(B) and (C)). However, at 27 d in culture, the cells showed a spindle-shaped cell morphology (figure 1(A)) and the expression of granulosa cell markers decreased, while the theca cell or luteal cell marker (LHR) increased (figure 1(D)). This indicates that phenotype of the ovarian cells can be changed during the traditional in vitro 2D culture [22–24]. Thus, the establishment of the 3D culture system of ovarian follicles is required to examine ovarian physiology and to define infertility treatments of or germline preservation. 3D culture of ovarian follicles could efficiently support the maintenance of their morphology and phenotypes and the interactions between cell–cell and cell–matrix [8, 25].

3.2. Collagen hydrogel characterizations

We used collagen hydrogels as an artificial microenvironment for a 3D culture system because the connective tissue within the ovary is mainly composed of collagen with a higher concentration at the ovarian surface epithelium and in the ovarian follicular compartment [26]. To observe the ultrastructure of the collagen hydrogels, SEM images were taken (figure 2(A)). As the concentrations of the collagen
hydrogels increased, the space between the collagen fibers became narrow and compact. As such, a collagen concentration of 7 mg ml\(^{-1}\) produced a denser and compact matrix with thinner fibers when compared to the lower concentrations of collagen.

The elasticity of the collagen hydrogels was varied by adjusting the solution concentrations. A series of collagen hydrogels were identified by the dynamic modulus of elasticity (E), which was measured by the rheometer. The elastic moduli of 60 Pa, 457 Pa, 1350 Pa, and 1671 Pa corresponded to the collagen hydrogel concentrations of 1, 3, 5, and 7 mg ml\(^{-1}\), respectively (table 1 and figure 2(B)). We hypothesized that this 3D culture system could provide mechanical support to the follicle; therefore, it could maintain cellular interaction and provide signals that promote follicle development and maturation [27, 28]. Moreover, the hydrogel elasticity is one of the key elements that can directly affect the phenotype and function of the in vitro-cultured ovarian follicles. The hydrogel should provide sufficient stiffness to maintain the 3D configuration of the follicle, yet it should also provide for an increase in size due to oocyte growth, ovarian stromal cell proliferation, and antrum development [20, 21, 29, 30]. Forces are produced by follicles in 3D structure when the expanding follicle exerts an outward force on the matrix.

### 3.3. Ovarian follicle viability and morphology

Ovarian follicles were cultured in the hydrogels made from varying concentrations of collagen hydrogels (1–7 mg ml\(^{-1}\)) in order to determine the optimal

![Figure 3. Ovarian follicle viability and diameter. (A) Microscopic, live/dead staining, and SEM images of ovarian follicles at 7 d. (B) Percent survival of the ovarian follicles cultured in different collagen hydrogels. (C) Measurement of diameter of the ovarian follicles cultured in varying concentrations of collagen. Data for the represent mean ± S.D. (n = 5). *P < 0.05 and **P < 0.01.](image)

![Figure 4. Hormone production from the culture ovarian follicles in collagen hydrogels with different concentrations. (A) 17β-estradiol and (B) progesterone.](image)
concentration to provide a suitable matrix elasticity for the follicular development such that their 3D structure and the capacity to produce mature oocytes was maintained. The viability of the ovarian follicles in the collagen hydrogels was determined using a live/dead staining assay (figure 3). In 1 mg ml\(^{-1}\) of collagen hydrogel, the viability of the ovarian follicles was the lowest at 40 \(\pm\) 35\%. In 3 mg ml\(^{-1}\) and 5 mg ml\(^{-1}\) of the collagen hydrogels, however, the viability of the ovarian follicles was over 90\% (90 \(\pm\) 4\% and 98 \(\pm\) 2\%, respectively). Interestingly, in 7 mg ml\(^{-1}\) of the collagen hydrogel, it decreased to 76 \(\pm\) 10\%.

Follicles grown in the 1 mg ml\(^{-1}\) of collagen hydrogels resulted in the development of large ovarian cells that tended to migrate away from the follicle (figure 3(A)). With increasing collagen concentrations, the size of follicles decreased and they seemed to be more closely associated with the follicle. Ovarian follicle growth was examined by measuring the follicle size at 0 d and 12 d in culture (figure 3(C)). At 0 d, the average diameters of the follicles were 89 \(\pm\) 21 \(\mu\)m, 87 \(\pm\) 14 \(\mu\)m, 95 \(\pm\) 31 \(\mu\)m, and 88 \(\pm\) 25 \(\mu\)m in 1, 3, 5, and 7 mg ml\(^{-1}\) collagen hydrogels, respectively. At 12 d in culture, the follicle diameter increased to 158 \(\pm\) 41 \(\mu\)m in 1 mg ml\(^{-1}\) of collagen hydrogel. In 3, 5, and 7 mg ml\(^{-1}\) of collagen hydrogels, the follicles had significantly increased in diameter to 252 \(\pm\) 59 \(\mu\)m, 267 \(\pm\) 66 \(\mu\)m, and 226 \(\pm\) 95 \(\mu\)m, respectively. In addition, the diameter of the follicles grown in the 5 mg ml\(^{-1}\) collagen hydrogel increased significantly compared to those in the 7 mg ml\(^{-1}\) of collagen hydrogel at 12 d (\(P < 0.05\)).

The matrix elasticity was increased as the concentration of collagen hydrogel increased, resulting in greater tie contact between fibers. We expected that a low concentration of collagen hydrogel (1 mg ml\(^{-1}\)) having large pore structure between fibers would be better for the ovarian follicle growth and maturation. However, the follicles grown in 3 mg ml\(^{-1}\) and 5 mg ml\(^{-1}\) of the collagen hydrogels showed more than 90\% cell viability, and these concentrations were significantly higher than the survival of follicles cultured in 1 mg ml\(^{-1}\) of collagen hydrogel. In addition, the cultured follicles in 3 and 5 mg ml\(^{-1}\) of collagen hydrogels increased in size to more than 250 \(\mu\)m, which was significantly bigger than those cultured in 1 mg ml\(^{-1}\) of collagen hydrogel. SEM analysis from follicles grown in 1 mg ml\(^{-1}\) of collagen hydrogel indicated that the ovarian cells easily migrated through the open structure of the gel construct and were not able to support normal follicle development. Follicles grown in 7 mg ml\(^{-1}\) collagen had a modest follicle survival (76\%) and a fairly large follicle diameter (226 \(\mu\)m). However, the follicles were smaller than the follicles grown in 3 mg ml\(^{-1}\) and 5 mg ml\(^{-1}\) of collagen hydrogels.

### 3.4. Production of 17\(\beta\)-estradiol and progesterone

On these porous and flexible supports, expression of specific features, initiation of maturation, and a greater sensitivity to hormone stimulation have been reported [31, 32]. In the hormone production, 17\(\beta\)-estradiol hormone is produced by the mature ovarian follicle and maintains oocytes in the ovary. While recent studies of in vitro folliculogenesis showed very encouraging data, the follicular growth rate was faster in vitro compared to in vivo-grown counterparts [20, 33]. Thus, 17\(\beta\)-estradiol and progesterone were detected in the conditioned media collected from cultured follicles in the collagen hydrogels (figure 4). 17\(\beta\)-estradiol levels differed significantly among all different collagen concentrations as early as 6 d of culture. In 5 mg ml\(^{-1}\) of the collagen hydrogel, the production of 17\(\beta\)-estradiol increased until 14–16 d, however, in 3 mg ml\(^{-1}\) and 7 mg ml\(^{-1}\) of collagen hydrogels, after 12 d, the production of 17\(\beta\)-estradiol decreased (figure 4(A)).
3.5. Oocyte meiotic competence facilitated follicular growth and maturation. Lagen hydrogels maintained the estrogen secretion and the results indicated that the 3D culture using the collagen hydrogels with different concentrations during pregnancy, in the cultured conditioned media. Natural female hormone that is essential before and after fertilization is progesterone, a hormone. Furthermore, we detected progesterone, a natural female hormone that is essential before and during pregnancy, in the cultured conditioned media. The results indicated that the 3D culture using the collagen hydrogels maintained the estrogen secretion and facilitated follicular growth and maturation.

Progesterone levels plateaued at 3 d and remained at that level through 16 d (figure 4(B)). Progesterone levels did not differ significantly with all collagen concentrations.

Our data indicated that immature ovarian follicles progress to mature form and secrete 17β-estradiol hormone. Furthermore, we detected progesterone, a natural female hormone that is essential before and during pregnancy, in the cultured conditioned media. The results indicated that the 3D culture using the collagen hydrogels maintained the estrogen secretion and facilitated follicular growth and maturation.

### 3.5. Oocyte meiotic competence

Oocyte quality produced from engineered follicles in the different concentration of collagen hydrogels was measured by their ability to continue meiosis, as shown by germinal vesicle breakdown (GVBD) (table 2). After 12 d in culture, oocytes from follicles cultured in 1 mg ml⁻¹ of collagen hydrogel demonstrated the lowest rate of meiotic resumption (34%, table 2). In 5 mg ml⁻¹ of the collagen hydrogel, oocytes showed highest percentage (88%, table 2) of GVBD. The percentage of GVBD differed significantly between collagen concentrations in 1 mg ml⁻¹ and 5 mg ml⁻¹. The isolated ovarian follicles had two to three layers of ovarian cells at 0 d (figure 5(A)). The follicles formed spherical antral follicle at 7 d (figures 5(C) and (D)) and dramatically ovulation of metaphase-II oocytes after 12 d in culture (figures 5(E) and (F)).

Human follicles cultured in 3D collagen hydrogel experienced follicular growth, whereas human follicles cultured on a 2D collagen coated surface only preserved their initial size and did not grow [34]. A report describing collagen encapsulation of follicles did not report on the quality of the resulting oocytes, and no antral follicles were seen after 14 d of culture using that system [35]. However, in this study, 50–72% of the oocytes produced in the 3D collagen cultures were meiotically competent at 12 d. Follicles grown in 5 mg ml⁻¹ of collagen hydrogel resulted in the highest number of GVBD stage mature oocytes, and this was followed by those grown in 7 mg ml⁻¹, 3 mg ml⁻¹, and 1 mg ml⁻¹ of collagen hydrogels. The biomaterial must present the needed signals for follicle maturation. Hormones and growth factors can be added to the culture medium and be transported through the hydrogel.

**In vivo**, the ECM regulates ovarian follicular function and differentiation. Providing an appropriate ECM in an in vitro culture system provides for a logical approach to cell culture and could lead to a better understanding of factors required for maintaining the quality of the follicle in culture [20, 36]. The addition of ovarian derived ECM in the 3D matrix may provide the growth factors needed to stimulate the follicle growth and maturation. Note that the adhesiveness of the hydrogel must be sufficient to limit cell migration out of the follicle, which can disrupt the structure of the follicle and affect its growth potential. Therefore, defining the structural and biochemical properties of the ECM for each stage of follicle development will facilitate the growth of ovarian follicle cells for female infertility due to cancer therapies, ovarian tissue bank, or fertility preservation. This study explores how the 3D microenvironment utilized by collagen hydrogels affects phenotypes of the in vitro-cultured ovarian follicles. Primary ovarian follicles cultured in collagen hydrogels with an elastic modulus of 1351 Pa (at 5 mg ml⁻¹) were more functional than cells in other concentrations of collagen hydrogels as shown by hormone secretion and oocyte maturation [33, 37, 38]. This work supports the notion that the proper microenvironment represents an important inducer of phenotypes in primary ovarian follicles.

### 4. Conclusions

We investigated how the collagen hydrogels of varying the concentration in the 3D culture system affected the ovarian follicle survival and development, including phenotypic maintenance, hormone production, and maturation and ovulation. These matured ovarian follicles could be useful for fertilization or preservation. Future studies will need to be established for each stage of follicle growth environments to obtain normal mature oocytes, in vitro fertilization, and development to normal offspring.

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