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| Original Article |

# Effects of Alginate Hydrogels on *In Vitro* Maturation Outcome of Mouse Preantral Follicles

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**Abstract :** Ovarian follicle growth and oocyte maturation require communications between the oocyte and its surrounding somatic cells. Three-dimensional structures provide cell culture conditions that increase interactions between the cellular components when compared to 2-dimensional structures. In this study, alginate matrices were employed to maintain a 3-dimensional architecture to examine its effects on *in vitro* culture outcomes using mouse ovarian follicles. Ovaries were obtained from the 14-day-old C57BL/6 mice, and preantral follicles were isolated and cultured in either a 2-dimensional culture system or 0.125/0.25% alginate matrices-based 3-dimensional culture systems. We successfully observed encapsulation within 0.125 and 0.25% alginate matrices, which maintained the follicular spherical architecture, with a centrally placed oocyte and layers of granulosa cells. All culture outcomes were comparable between the 2-dimensional control and 3-dimensional alginate group. However, 0.25% alginate hydrogels led the lower rates of follicle survival and antral formation in comparison to the 2-dimensional control and 0.125% alginate. These results demonstrate that a non-permissive stiffness from the high concentration of alginate could interfere with cell-to-cell communication of oocytes and follicles. The extracellular matrix interacts with the oocyte and granulosa cells, resulting in direct effects on their proliferation. The development of a 3-dimensional culture system can enhance follicle maturation and produce meiotically-competent oocytes to provide advanced reproductive options for IVF patients in the future.

**Key words:** *alginate, extracellular matrix, follicle, ovary, in vitro maturation*

## 1. Introduction

Recently, *in vitro* follicle maturation has been developed and applied as a fertility treatment for women diagnosed with polycystic ovary syndrome in order to decrease the rate of ovarian hyperstimulation and also to preserve fertility in patients facing fertility-threatening disease or treatment regimens such as chemotherapy.[1-3] Ovarian follicle maturation is a complicated process involving the interaction of auto- and paracrine factors and systematic endocrine signals. With respect to developing an *in vitro* culture system, the production of mature competent oocytes presents many challenges. In turn, various culture systems have been applied to *in vitro* follicle maturation to provide optimal conditions for proper oocyte maturation.

The ovarian follicle consists of an oocyte surrounded by layers of granulosa cells, a basement membrane composed of extracellular matrix (ECM), and an outer layer of theca cells. As follicles develop, the somatic cells surrounding the oocyte proliferate and differentiate, leading to the maturation of the oocyte. Communication between the multiple cellular components of the follicle is essential for its development and oocyte maturation. Previous studies indicated that gap junctions between the oocyte and its surrounding granulosa cells are critical for paracrine communications and the transport of specific amino acids to the oocyte.[4-6] However, in existing 2-dimensional culture formats, this communication is disrupted by the flattening of the follicle as its somatic cells spread away from the oocyte. Three-dimensional culture systems mimic the *in vivo* environment more faithfully than 2-dimensional systems.[7,8] In addition to maintaining cell-to-cell communication, cells in 3-dimensional culture systems utilize different integrins, which are important for ECM attachment and support

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of the cellular architecture.[9] Likewise as 3-dimensional structures, follicles should develop more favorably in a 3-dimensional culture system.

Alginate follicle culture systems have been investigated to provide a 3-dimensional scaffold matrix for supporting the growth of multilayered follicles.[7,8,10,11] Alginate is a widely used biomaterial that has numerous applications in biomedical science and tissue engineering[12], and is suitable for follicle culture due to its gelling physical properties.[13,14] Alginate that is produced by brown algae is a linear polysaccharide copolymer of β-D-mannuronic acid and α-L-guluronic acid[15] and forms a mesh-like structure that allows for diffusion of essential hormones and other proteins such as follicle stimulating hormone (FSH).[7] By using an alginate follicle culture system, meiotically competent oocytes can be produced and fertilized successfully, resulting in the production of viable offspring.[16]

We investigated the optimal concentration of alginate hydrogels for *in vitro* 3-dimensional culture of mouse preantral follicles. Although 3-dimensional culture systems are theoretically superior to 2-dimensional culture systems, previous reports have failed to achieve typical follicle sizes observed *in vivo* and failed to form proper antral follicles and theca cell differentiation while using alginate encapsulated follicle culture systems.[7,8,10] Some investigators suggested that the physical properties of the matrix may influence follicle development and showed that decreasing the matrix stiffness enhances follicle growth as evidenced by antral formation, theca cell differentiation, oocyte maturation, and hormone production levels.[15,17] Herein, we investigated the effect of alginate consistency on oocyte developmental competence of encapsulated follicles in alginate scaffolds of various concentrations.

## 2. Materials and Methods

### 2.1. Isolation of Mouse Preantral Follicles

We isolated preantral follicles from 14-day-old female C57BL/6 mice. The mice were sacrificed via cervical dislocation and the ovaries were isolated by dissection ovaries. Early preantral follicles were mechanically dissected from the ovaries using 28 gauge needles plunged in MEM media. All experimental procedures were performed under the provisions of the Institutional Animal Care and Use Committee of Seoul National University Hospital.

### 2.2. Preparation of Encapsulation

1.2% sodium alginate solution (Lonza, Walkersville, MD, USA) was diluted with sterile 1x phosphate buffered saline. 0, 0.125 and 0.25% alginate solutions were used to encapsulate follicles. Droplets were formed with 60 μl of alginate solution placed onto 8 μM pore size mesh (Nunc, Roskilde, Denmark) and a single follicle was inserted in each drop. The mesh was immersed in a solution containing 50 mM of $CaCl_2$ (Sigma, St. Louis, MO, USA) and 140 mM NaCl (Sigma, St. Louis, MO, USA) for 2 minutes at room temperature to crosslink the alginate.

### 2.3. *In Vitro* Culture of Follicle

Non-coated or alginate-coated follicles were placed in 20 μl of culture media and half of the media volume was exchanged every 2 days. Culture media consisted of MEM alpha (Invitrogen, Grand Island, NY, USA), 5% FBS (Hyclone, Morgan, Utah, USA), insulin-transferrin selenium (Invitrogen) and penicillin/streptomycin (Invitrogen), supplemented with recombinant follicle stimulating hormone (FSH) and luteinizing hormone (LH). The selected preantral follicles were cultured in a culture dish and incubated at 37°C in 5% $CO_2$ for up to 12 days. Follicle growth was observed under the phase-contrast microscope daily. Following 12-14 days *in vitro* culture, human chorionic gonadotropin (hCG) was added to induce ovulation.

### 2.4. Measurement of Follicle Growth

Follicle diameter in each group was measured every other day during culture under phase-contrast microscope and calculated using i-solution software (InnerView, KyungGi-do, Korea). Means and standard deviations were compared using Student's *t*-test and chi-square test. Differences were considered statistically significant when $p < .05$.

## 3. Results

### 3.1. Effects of Alginate on Follicle *In Vitro* Growth

To encapsulate the 25 follicles with the alginate hydrogel, we used cell culture mesh to coat many cells at once. A schematic illustration of the encapsulation apparatus is represented in Fig 1.

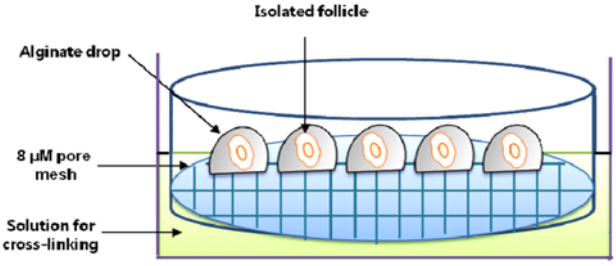


Figure 1. Apparatus for encapsulation of a follicle.
Mesh with large pore is used as sieve for the fast and large volume of alginate coating.

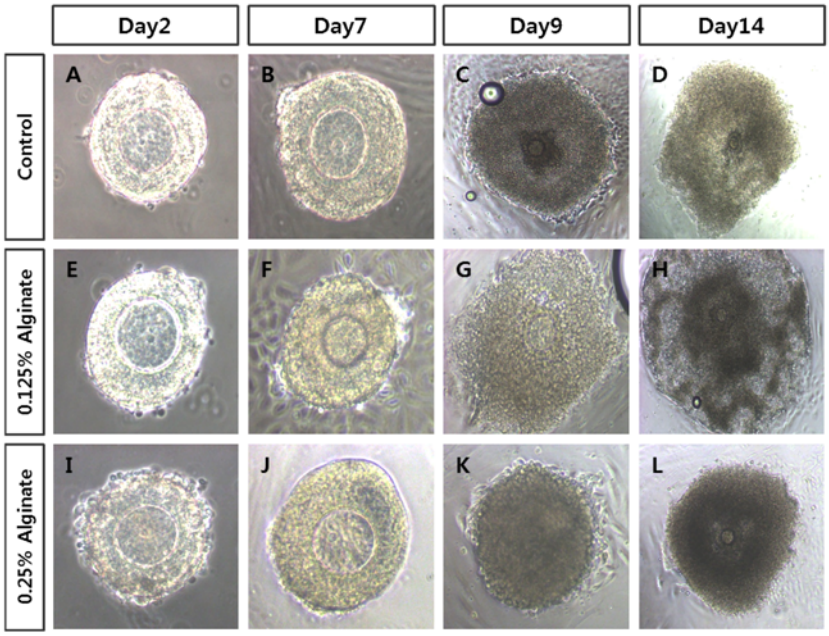


**Figure 2.** Development of preantral follicles during *in vitro* culture. Typical follicle morphology of each group at day 2, 7, 9, 14 during *in vitro* culture are shown.

We evaluated the effect of the hydrogel during *in vitro* culture at three different concentrations, 0, 0.125 and 0.25% on follicular morphology (Fig 2). However, 0.25% alginate hydrogels led the lower rates of follicle survival and antral formation in comparison to the 2-dimensional control and 0.125% alginate.

The mean diameter of Day 2 follicles was shown no statistical significant difference among the three groups. Day 14 follicles showed noticeable differences in diameter between each of the groups, with the difference being most pronounced between the 0.125% and 0.25% alginate groups (Fig 3).

To assess the effect of alginate on follicular growth, we also calculated the percentage of degenerated follicles of the groups at Day 14 (Table 1). The rate of degenerated follicles was significantly greater in both alginate-encapsulated conditions compared to the control group. The rate difference between 0.125% and 0.25% groups, however, was smaller than the difference between the control and alginate-encapsulated group.

**Table 1.** Degeneration rates of cultured follicles in three groups.

| | CTL | 0.125% Alginate | 0.25% Alginate |
|---|---|---|---|
| #1 | 2% | 40% | 68% |
| #2 | 44% | 55% | 77% |
| #3 | 38% | 64% | 66% |
| #4 | 48% | 72% | 75% |

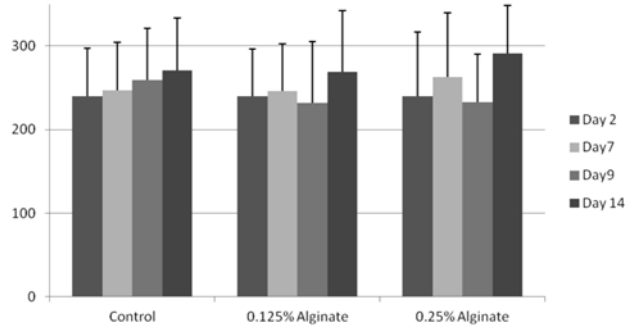


**Figure 3.** Diameters of cultured follicles in three groups.

These results show the rate of degenerated follicle increased in correlation to concentration of alginate hydrogel and alginate encapsulation, therefore may not be optimal for *in vitro* culture of follicle, at least in our condition.

## 4. Discussion

Three-dimensional culture systems may be necessary for proper ovarian follicle development *in vitro* due to the complex interactions that occur between the oocyte and surrounding somatic cells via communication through gap junctions. Encapsulation of ovarian follicles is an approach to maintain the follicle's 3-dimensional architecture, as this system not only

the prevents gap junction disruptions but also increases the concentration of trophic factors in the immediate vicinity of the growing oocyte. The alginate model has been the most commonly studied 3-dimensional cell culture system and has contributed much to our understanding of follicle cell biology.[10,15,18-20] Alginate seeded with preantral follicles can be formed into small beads that are 500-1,000 μm in diameter through cross-linking in the presence of calcium. Alginate encapsulated follicles are responsive to FSH stimulation, secrete steroid hormones, express gap junction proteins, and yield mature oocytes capable of being fertilized and resulting in live offspring.[7,8,15,16,18-23] Although previous studies have generated much positive data in support of the benefits of alginate-based follicle culture and the development of morphologically-accurate oocytes, a recent study suggested that alginate-based cultures may introduce disturbances to meiotic spindle assembly.[22] The investigators proposed this negative effect may contribute to lower developmental competence in oocytes derived from the alginate culture system.

Other studies have revealed that the stiffness and density of the alginate matrix play a crucial role in determining the level of enhancement experienced by follicular development in an alginate-based 3-D culture system.[15,17] Decreasing the percentage of alginate in solution decreases the shear elastic modulus and facilitates diffusion of molecules through alginate. With 3-dimensional culture systems, adjusting the rigidity of the matrix may be vital for granulosa cell expansion and antrum formation.[15,17] To optimize the concentration of alginate, we compared follicle developmental outcomes at different alginate concentrations. We referred to previous studies and found 0.25% of alginate was the most extensively tested concentration that led to favorable results.[10,15,19,24,25] Therefore, in this study we decided to compare the culture outcomes between control and groups consisting of 0.125 and 0.25% alginate hydrogels as the matrix scaffold.

We observed successful follicular encapsulation within 0.125 and 0.25% alginate matrices. The encapsulated follicles maintained a spherical architecture with a centrally placed oocyte and surrounding layers of granulosa cells. Nonetheless, our study revealed increased rates of follicular degeneration and significantly smaller follicular diameters in the 0.25% alginate group when compared to the control and 0.125% alginate matrix. These results are consistent with previous studies that demonstrated higher concentrations of alginate deteriorate the culture outcomes as measured by antral formation, theca cell differentiation, oocyte maturation and steroid hormone production.[7,8,10,15,17,22] However, with regard to demonstrating the advantages a 3-dimensional culture system has over a 2-dimensional culture system, our present study found opposing results to previous investigations. In our prior unpublished data using a 2-dimensional follicle culture system, follicle survival rate was 82% and antral formation rate was 76%. In this study, the alginate-based 3-dimensional follicle culture system showed much lower rates of follicle survival and antral formation compared to our 2-dimensional culture method. Our results reflect the notion that the non-permissive stiffness caused by a high concentration of alginate may interfere with cell-to-cell communication and negatively affect the proliferation of oocytes and follicles.

To understand the differences between the 2-dimensional culture control group and the alginate-based 3-dimensional culture group, further studies will be necessary to establish the relative importance of cell-to-cell communication in oocyte-granulosa cell components.

The regulation of follicle development has been suggested to be primarily controlled by the oocyte,[26] as oocyte derived factors directly influence granulosa cell function and follicle maturation. Our study demonstrates that a stiffer 3-dimensional culture environment caused by higher concentrations of alginate negatively affects the differentiation of multiple somatic cells and in turn indicates that the extra-follicular ECM milieu also affects follicular development, which suggests a new factor of regulation in *in vitro* follicle culture systems. Though our results cannot directly explain the effect of cell-to-cell communications, our results hint at the notion that the ECM interacts with the oocyte and granulosa cells, resulting in direct effects on their proliferation. These results may lead to new clinical treatment options for *in vitro* ovarian follicular maturation and serve as a topic for future tissue engineering studies.

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