Encapsulation of bovine primordial follicles in rigid alginate does not affect growth dynamics

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Background

Premature ovarian insufficiency (POI) results from the depletion of the ovarian reserve to less than 1,000 primordial follicles before the age of 40. There are many causes of POI, including idiopathic, genetic, autoimmune, and iatrogenic. Ovarian tissue cryopreservation (OTC) is the only pre-treatment fertility preservation option available to patients who cannot ovulate or for those whom ovarian stimulation and egg retrieval are not advisable. The OTC process includes the surgical removal of an ovary and cryopreservation of the ovarian cortex for future autotransplantation. The success rate and functional lifespan of the transplanted tissue varies significantly with only 20–40% of patients having a successful pregnancy after transplant and the functional duration of the transplanted tissue ranging from 2 months to 12 years. There is a mass activation event following transplantation that results in an 80% reduction in primordial follicles which make up the ovarian reserve. To quell this mass activation event and optimize current autotransplantation techniques, a better understanding of the factors that influence primordial follicle activation and quiescence is needed. New ovarian fertility restoration techniques are desperately needed that support long-term restoration in a safe way, especially for those that have metastatic disease. Additionally, transgender individuals and those with differences of sexual development may undergo elective or medically necessary gonadectomy and decide to cryopreserve and save tissue that contains germ cells. This population would also benefit from alternative restoration technologies. A bioprosthetic ovary made of isolated follicles in a three-dimensional printed gelatin scaffold restored hormone production and fertility in ovariectomized mice. This approach could potentially allow follicles to be isolated from any metastatic tissue prior to transplantation, enhancing the safety of fertility restoration. An engineered bioprosthetic scaffold that incorporates controls for primordial follicle activation would also improve graft longevity. Primordial follicles, which reside in the stroma and develop ovarian cortex, grow better when encapsulated in different environments (Figure 1). Disruption of the extracellular matrix (ECM) in murine ovaries increased primordial follicle activation, while exogenous physical pressure added to these ovaries restored primordial follicle quiescence. In our study, we sought to investigate how differences in encapsulating rigidities would affect the quiescence and growth of isolated or in situ bovine primordial follicles. We further explored the impact of isolating primordial follicles from bovine ovarian cortex tissue pieces on the determination of the isolation process as an irreversible activation event within follicles. Our findings contribute to the understanding of primordial follicle activation and will inform the development of fertility restoration techniques.

Methods

Prior to experiments, randomly selected bovine ovaries were removed from the left ovaries of two 4-month-old bovine cows and prepared for in vitro culture. Ovarian cortex tissue pieces were then either encapsulated in 1% or 5% alginate gel beads. Bovine cortex tissue samples were reserved at −80°C until use. Tissue in panel A was incubated with EdU immediately after the ovarian cortex was removed using the tissue slicer. Tissue in panel B was incubated immediately after processing by the tissue chopper. Tissue in panel C was incubated after enzymatic digestion. Tissue in A was co-stained for Mv52 using immunofluorescence techniques. Isolated follicles in panel D were incubated immediately after mechanical isolation and follicles in panel E were incubated 1 day after culture in white arrows. Yellow arrows pointing to follicles stained red with Mv52 in the tissue. Blue arrows pointing to stromal cells stained green in the tissue indicating EdU incorporation in cells away from follicles. Yellow arrows pointing to isolated follicles with green stained granulosa cells indicating EdU incorporation. White scale bars are 100µm and the red scale bars are 10µm.

Results


Figure 2. The schematic outlines the process of isolating and culturing both bovine cortical tissue pieces and follicles in 1% and 5% alginate gel beads. Bovine primordial follicles were isolated from ovarian cortex. A medium of 10 follicles (range 5-24) were encapsulated per bead in either 1% or 5% alginate gel beads. Bovine ovaries were obtained within 24 hours of animal sacrifice and then dissected (1). Ovaries were then sliced into 0.5mm thick slices using a Stadie-Riggs Tissue Slicer (2). Tissue slices were then further processed into 1 by 1 by 0.5mm pieces using a tissue chopper (3-4). Tissue pieces were then either encapsulated in alginate gel for in situ experiments (9) or underwent enzymatic and mechanical digestion (4-5). The sample was then passed through a cell strainer and follicles were eluted using a holding media (6). Isolated follicles were then crosslinked in either 1% or 5% alginate (7). The encapsulated follicles and tissue were subsequently crosslinked in a calcium sulfate solution (8). Follicles were then cultured for 8 days with light microscopy images taken every other day along with media exchanges (8). Growth and survival curves were constructed. In situ experiments were cultured for 0, 4, and 8 days. Cortex tissue was then submitted for histologic processing and HE staining (10). Follicles were then counted and staged. A total of 12 pieces of ovarian cortex were analyzed for each condition on each day. All statistical analyses were performed using Graph Pad Prism 9.

Figure 3. Growth and survival of primordial follicles encapsulated in differently rigid alginate gels. (A) Primordial follicles encapsulated in 1% and 5% alginate were examined under light microscopy every other day for all 8 days of culture. The same representative follicle is shown over time from each culture condition. Scale bars are 100µm. (B) Growth curves were plotted using mean ± SEM of primordial follicle diameter. A multivariable regression utilizing day, number of follicles encapsulated per bead, and alginate concentration was constructed to analyze the effects of these covariates on follicle diameter. Survival curves between the two concentrations of alginate were also constructed. (C) After 8 days in culture, the presence of bovine follicles was stained for DDX4 and Mv52 using whole mount immunofluorescence techniques.

Figure 4. Evaluating survival of follicles grown in situ in differentially rigid conditions. (A) Tissue was cultured for up to 8 days in either no gel, 1% alginate, or 5% alginate. Alginate is marked with yellow stars. (B) Representative images of follicles found in situ tissue that was cultured for up to 8 days. Primordial, primary and secondary follicles are noted by green stars. Scale bars are 50µm. (C) Total and primordial follicles were counted in tissues fixed on days 4, 8 and 12 to assess for follicle numbers in cultures without gel, in 1% alginate, and in 5% alginate. The means SEM are graphed for each condition and timepoint.

Figure 5. EdU uptake during tissue processing for primordial follicle isolation. Bovine cortex tissue samples were reserved at specific time points during the ovarian tissue processing and primordial follicle isolation process for EdU incorporation. Tissue in panel A was incubated with EdU immediately after the ovarian cortex was removed using the tissue slicer. Tissue in panel B was incubated immediately after processing by the tissue chopper. Tissue in panel C was incubated after enzymatic digestion. Tissue in A was co-stained for Mv52 using immunofluorescence techniques. Isolated follicles in panel D were incubated immediately after mechanical isolation and follicles in panel E were incubated 1 day after culture in white arrows. Yellow arrows pointing to follicles stained red with Mv52 in the tissue. Blue arrows pointing to stromal cells stained green in the tissue indicating EdU incorporation in cells away from follicles. Yellow arrows pointing to isolated follicles with green stained granulosa cells indicating EdU incorporation. White scale bars are 100µm and the red scale bars are 10µm.

Conclusions

- Isolating primordial follicles is an activating event that is not affected by rigid environments.
- Primordial follicle quiescence likely relies on multiple cues, both physical and biochemical.
- More work is needed to better understand each of these cues and how they relate to each other to understand primordial follicle activation and its role in premature ovarian insufficiency and to develop future fertility preservation technologies.

References


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