A new step toward the artificial ovary: survival and proliferation of isolated murine follicles after autologous transplantation in a fibrin scaffold

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Objective: To create an artificial ovary to provide an alternative way of restoring fertility in patients who cannot benefit from transplantation of cryopreserved ovarian tissue due to the threat of reintroducing malignant cells.
Design: In vivo experimental study.
Setting: Gynecology research unit in a university hospital.
Animal(s): Six-week-old female NMRI mice.
Intervention(s): Autografting of isolated preantral follicles and ovarian cells (OCs) encapsulated in two fibrin matrices containing low concentrations of fibrinogen (F; mg/mL) and thrombin (T; IU/mL): F12.5/T1 and F25/T4.
Main Outcome Measure(s): Follicular density and development, OC survival and proliferation, inflammatory response, and vascularization.
Result(s): After 1 week, the follicle recovery rate ranged from 30.8% (F25/T4) to 31.8% (F12.5/T1). With both fibrin formulations, all follicles were found to be alive or minimally damaged, as demonstrated by terminal deoxynucleotide transferase–mediated dUTP nick-end labeling assay, and at the growing stage (primary, secondary, and antral follicles), confirmed by Ki67 immunostaining. Isolated OCs also survived and proliferated after grafting, as evidenced by $<1\%$ apoptotic cells and a high proportion of Ki67-positive cells. Vessels were found in both fibrin formulations, and the global vascular surface area varied from 1.35% (F25/T4) to 1.88% (F12.5/T1). Numerous CD45-positive cells were also observed in both F25/T4 and F12.5/T1 combinations.
Conclusion(s): The present study is the first to show survival and growth of isolated murine ovarian follicles 1 week after autotransplantation of isolated OCs in a fibrin scaffold. The results indicate that fibrin is a promising candidate as a matrix for the construction of an artificial ovary. Xenotransplantation of isolated human follicles and OCs is the necessary next step to validate these findings. (Fertil Steril® 2014;101:1149–56. ©2014 by American Society for Reproductive Medicine.)
Key Words: Preantral follicles, artificial ovary, fibrin, ovarian cells, cancer patients, fertility preservation, transplantation

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Cancer is a growing global problem, and much research is being conducted to understand the mechanisms of the disease and to develop effective treatments. However, some cancer treatments, especially alkylating agents and total body irradiation, may induce premature ovarian failure and affect the ability to have children (1). Different options for fertility preservation are commonly proposed to cancer patients. Embryo and mature oocyte cryopreservation

are well established procedures to preserve fertility in women of reproductive age. On the other hand, ovarian tissue cryopreservation is the only possible way of preserving fertility in prepubertal patients and women who require immediate cancer treatment (1, 2). In the literature to date, 30 live births have been published after autotransplantation of frozen-thawed ovarian tissue (3). However, transplantation of cryopreserved ovarian tissue cannot be performed when there is a risk of reimplanting malignant cells, as seen in leukemia patients, for example (4–7). For women in whom transplantation of ovarian tissue is not advisable, new options need to be developed.

To avoid the risk of transplanting malignant cells, grafting of isolated preantral follicles could be a safer alternative, because a basement membrane surrounding the ovarian follicles separates them from the stromal environment, blood vessels, and nerves (8). Our previous studies demonstrate the viability of these follicles after mechanical and enzymatic isolation and their survival and growth potential after in vitro and in vivo experiments (9–14).

The main challenge now is to develop a biocompatible and biodegradable artificial ovary that maintains the three-dimensional (3D) structure of isolated ovarian follicles and allows their full development. This artificial ovary should also ensure good cell proliferation and differentiation, cell invasion, and vessel recruitment and thus create an optimal environment for the survival and growth of these preantral follicles, thanks to gradual degradation of the matrix (15). In a recent study (16), nine fibrin formulations containing different concentrations of fibrinogen and thrombin were tested. After in vitro culture of isolated human ovarian stromal cells, we observed that fibrin formulations with low fibrinogen and thrombin concentrations (12.5 mg/mL fibrinogen with 1 IU/mL thrombin [F12.5/T1] and 25 mg/mL fibrinogen with 4 IU/mL thrombin [F25/T4]) yielded better survival and proliferation of these cells. The aim of the present study was therefore to evaluate the survival and development of isolated preantral follicles encapsulated in these fibrin formulations to assess if they can be used as a matrix to construct a transplantable artificial ovary.

MATERIALS AND METHODS

Experimental Design

Seven series of experiments were carried out using ovaries from seven mice. Both ovaries from each mouse were removed for isolation of preantral follicles and ovarian cells (OCs). After isolation, groups of 50 isolated follicles and 50,000 isolated OCs were encapsulated in fibrin matrices and grafted to the peritoneal pocket of the same mouse. Follicles and cells in fibrin clots that were not grafted were processed for live/dead assays to evaluate cell viability before grafting. After 7 days of autotransplantation, the grafts were fixed and analyzed for follicle survival and development, OC survival and proliferation, and graft vascularization and healing.

Ovariectomy Procedure

Guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain. Nine 6-week-old female NMRI mice from the local animal facility were used for this study. Animal housing conditions were previously described by Vanacker et al. (13). Each mouse was ovariectomized with the use of the lumbotomy approach, and the ovarian bursa containing the ovary was removed and placed in minimal essential medium (MEM) + Glutamax (Gibco) at 4°C for further isolation. Fatty tissue surrounding the ovaries was removed with surgical scissors.

Isolation of Preantral Follicles and Ovarian Cells from Mouse Ovaries

The ovaries were cut into 0.5 × 0.5 mm pieces with the use of a tissue sectioner (McIlwain Tissue Chopper; Mickle Laboratory) adjusted to 0.5 mm. Minced ovarian tissue was then transferred to a 10-mL conical tube containing Dulbecco's phosphate-buffered saline (PBS) solution without calcium or magnesium (Lonza) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) at 4°C. The resulting suspensions were centrifuged at 50 *g* for 15 minutes at 4°C. The pellets were resuspended in PBS supplemented with 10% FBS, then pipetted to once again mechanically disrupt digested tissue. Supplemented PBS was used because it is colorless and renders follicle pick-up easier than media with pH indicators. The solution was transferred to Petri dishes and investigated for follicles under a stereomicroscope (Leica; Van Hopplynus Instruments). The whole isolation procedure took no more than 10 minutes. Follicles were picked up with the use of a 130-μm micropipette (Flexipet; Cook) over the course of 45 minutes (9, 10). Thereafter, the rest of the cell suspension was successively filtered through sterilized 80-μm and 11-μm nylon net filters (Millipore). OCs were counted with the use of trypan blue (Sigma-Aldrich) and a Bürker chamber (VWR). The filtered solution was centrifuged at 260 *g* for 5 minutes and the pellet resuspended in PBS supplemented with 10% FBS to obtain a concentration of 50,000 cells/3 μL.

Fibrin Clot Formation

Tissucol (Baxter) is a two-component fibrin sealant. When combined, the two components, namely sealer protein and thrombin, mimic the final stage of the blood coagulation cascade. The active ingredient in sealer protein (human) is fibrinogen. Both components were reconstituted and diluted as previously described (16). Fibrinogen (100 mg/mL) was reconstituted in a solution containing 3,000 KIU/mL bovine aprotinin, a fibrinolysis inhibitor, at 37°C. Thrombin (4 IU/mL) was reconstituted in 40 mmol/mL calcium chloride ($CaCl_2$) solution. The reconstituted fibrinogen was diluted in saline solution (0.9% NaCl) containing 9 g/L NaCl to obtain two final concentrations: 25 mg/mL and 12.5 mg/mL. Thrombin was diluted in 40 mmol/L $CaCl_2$ solution to obtain two final concentrations: 4 UI/mL and 1UI/mL.

A droplet of 12.5 μL fibrinogen was placed on a glass Petri dish, and 50,000 mouse OCs in 3 μL of medium were mixed with the fibrinogen droplet. Fifty isolated murine ovarian follicles in 2 μL medium were then added to the fibrinogen droplet with the OCs. This droplet, containing isolated murine ovarian follicles and OCs, was mixed with a 12.5-μL volume of thrombin on a