Complete follicular development and recovery of ovarian function of frozen-thawed, autotransplanted caprine ovarian cortex

Frozen-thawed ovarian cortical fragments (1 mm^3) were autotransplanted to the uterus of completely ovariectomized goats. The grafts developed preovulatory follicles, accompanied by estrous behavior and a rise in plasma E_2 levels, demonstrating successful cryopreservation and transplantation. (Fertil Steril® 2008;■: ■–■. ©2008 by American Society for Reproductive Medicine.)

Cryopreservation of ovarian tissue has been advocated to preserve female gametes, particularly those in primordial follicles. Because complete in vitro development of follicles from large mammals currently is not feasible, transplantation is a more suitable method to acquire follicular development after freezing-thawing of ovarian tissue. Although ovarian cryopreservation is relatively successful in juvenile mice, efficient procedures have yet to be developed for adult women and livestock animals. Frozen-thawed ovine ovarian fragments transplanted onto the uterus displayed follicular growth reaching antrum formation within 13 weeks after transplantation. However, pregnant mare serum gonadotropin injection was required to induce estrus, and $>95\%$ of the follicles failed to survive (1). In the present study, goats were completely ovariectomized before transplantation of frozen-thawed ovarian tissue. Our aim was to validate the biologic function of this cryopreserved ovarian tissue.

The Institutional Animal Care and Use Committee of Utrecht University approved this study. Twenty-six adult, nonpregnant normal cycling goats were divided into three groups. In the first group, 5 goats were used as controls, that is, no surgery. In the second one, the fresh transplantation group, 11 goats were completely ovariectomized, after which ovarian fragments were grafted onto the uterus. In the third one, the frozen-thawed transplantation group, 10 goats were completely ovariectomized, and ovarian fragments were cryopreserved, thawed, and then autotransplanted. After premedication and anesthesia, a ventral midline skin incision was made to perform the complete ovariectomy. For transplantation, 15 ovarian cortical fragments (approximately 1 mm^3) were sutured together and grafted with Prolene 5/0 onto the curvature minor region of the uterine horn (left side). The same transplantation procedure was performed 14 days after ovariectomy for ovarian tissue that was frozen-thawed according to Santos et al. (2).

The study started at the end of autumn, and all animals were housed in a light-controlled room (3). Estrous behavior was evaluated daily (4). During the spring, 23 of the animals were killed for graft recovery, and three (one of each experimental group) were housed under natural light conditions.

Blood samples were collected thrice weekly from the beginning of the study till the three remaining animals were submitted to natural light, and blood collection was restarted at the end of the subsequent summer. Concentrations of P_4, E_2, and T were determined by a solid-phase RIA method (Coat-A-Count TKPG, TKE, and TKTT, respectively; Diagnostic Products Corp., Los Angeles, CA). Ovarian tissue was processed histologically and evaluated, follicles were classified as morphologically normal or degenerated (2), and follicular density was determined (5). The percentages of normal early stage follicles were compared by ANOVA and Tukey's test. Mean values of follicular density per square millimeter were compared by Student's *t*-test and ANOVA.

Cryopreserved ovarian cortical tissue fragments were morphologically similar to those of the controls, except that only early stage follicles (primordial up to and including secondary follicles) had survived the freezing-thawing. Grafted fresh ovarian tissue from one animal was recovered 1 week after surgery. In this transplant, blood vessels with erythrocytes were present, indicative of revascularization, and follicles that had survived the transplantation procedure were all primordial. One month after grafting, ovarian fragments had formed a single compact ovarian-like structure (Fig. 1A) in four of five goats with transplanted frozen-thawed cortical fragments and three of five animals that had received fresh ovarian cortical tissue. Approximately 3 months after grafting, in 80% (four out of five) of the animals that received frozen-thawed grafts and 60% (three out of five) of animals that received fresh ovarian fragments, the

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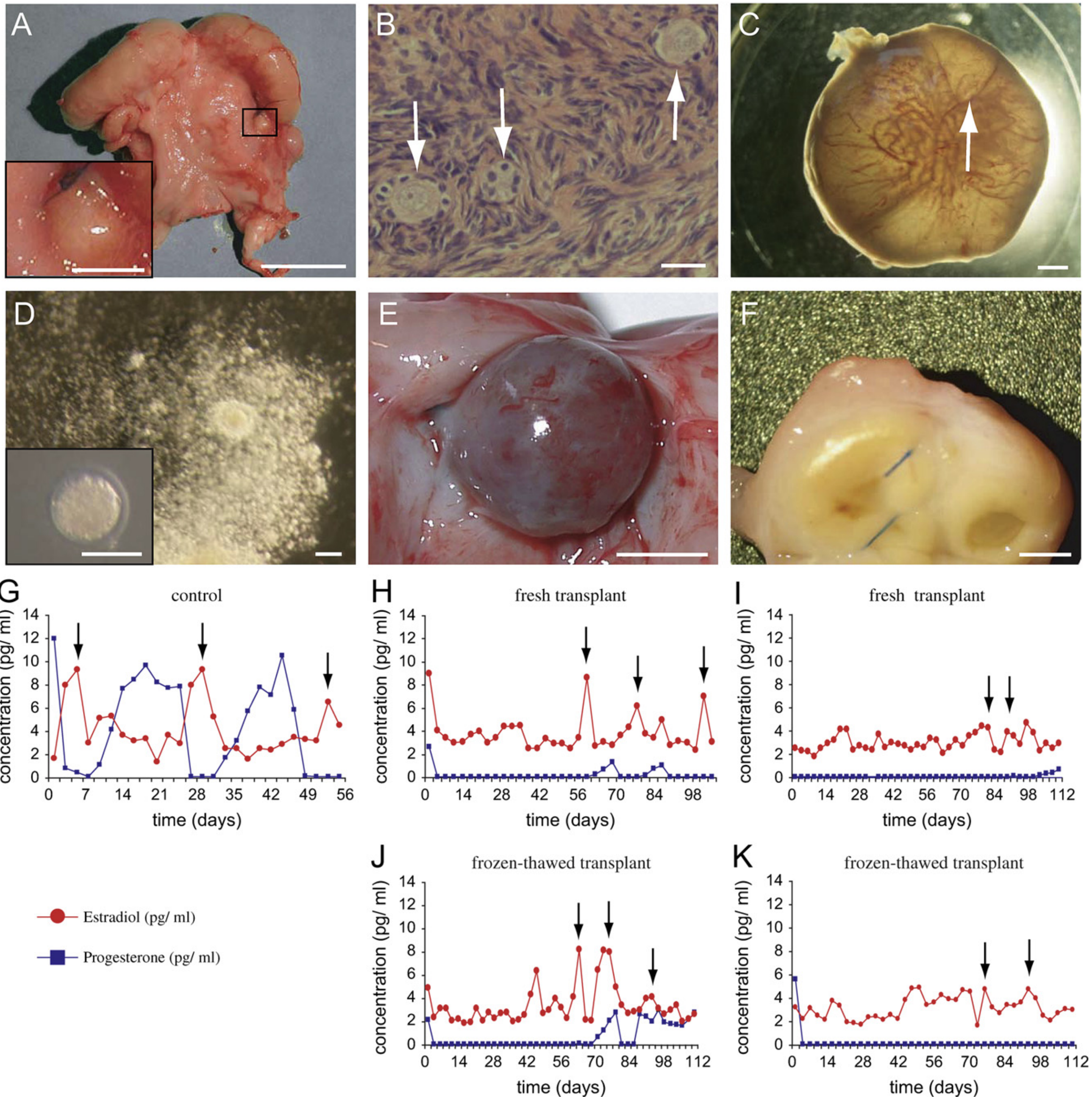
FIGURE 1

(**A**): Uterus with frozen-thawed ovarian tissue 1 month after grafting forming a compact ovarian-like structure (*inset* shows higher magnification), in which (**B**) primordial ovarian follicles started to develop to intermediate stages, indicative of activation (*arrows* indicate intermediate follicles).(**C**): Macroscopic view of a preovulatory follicle at 3 months after transplantation (*arrow* indicates blood vessels).(**D**): A mature cumulus-oocyte complex collected from a preovulatory follicle (*inset* shows a higher magnification of the denuded oocyte).(**E**): Macroscopic view of an active corpus luteum.(**F**): Longitudinal section of remodulated ovarian tissue with two corpora lutea that are poorly vascularized. *Scale bars*: (**A**): 5 cm (*inset*: 1 cm); (**B** and **C**): 100 μm; (**D** and **E**): 1 cm; (**F**): 1 mm; (**G** to **K**): Representative hormone profiles (*red line*: estrogen, picograms per milliliter; *blue line*: P, nanograms per milliliter) of control goat (**G**), goats that received fresh transplantation (**H** and **I**), and goats that received frozen-thawed ovarian tissue (**J** and **K**). *Arrows* indicate estrous behavior.



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recovered tissue contained, besides numerous early stage follicles (Fig. 1B), intact preovulatory follicles (Fig. 1C), from which cumulus-oocyte complexes were collected (Fig. 1D). Well-vascularized (Fig. 1E) and poorly vascularized (Fig. 1F) corpora lutea were also observed. Grafts collected 1 year after transplantation (both fresh and frozen-thawed) presented a preovulatory follicle with a mature oocyte and a corpus luteum, respectively. Percentages of normal follicles were not influenced by the posttransplantation collection period. A decrease in the percentages of normal early stage follicles was observed in goats after transplantation when compared with those of ovaries from control animals. The mean density of early stage follicles was $14.1 \pm 3.7/mm^2$ in control ovarian tissue and had significantly decreased to $8.2 \pm 2.1/mm^2$ and $7.5 \pm 3.6/mm^2$ in fresh and frozen-thawed transplanted tissue, respectively. Differences between the two transplantation groups were not statistically significant ($P>.05$). From the 10 goats of which ovarian tissue was collected 3 months after transplantation, estrous behavior was first detected at days 71.2 ± 7.9 and 71.8 ± 13.9 after transplantation of frozen-thawed and fresh ovarian tissue, respectively. Estrous behavior was accompanied by an increase in the plasma E_2 concentration (Fig. 1G to K). In two of five animals with frozen-thawed transplanted tissue and three of five animals with fresh-transplanted tissue, the rise in E_2 was not followed by a rise in P_4, corresponding with the absence of corpora lutea. Concentrations of T remained at control levels, that is, <0.1 ng/mL. All control animals were cycling during the whole experiment, except for the natural anestrous period in spring and summer, when animals presented only basal levels of E_2 and P_4 and no estrous behavior. Of each group (control, fresh transplanted, and frozen-thawed transplanted), one animal was kept until after the anestrous period. These animals presented estrus again paralleled by elevated plasma E_2 concentrations during that period.

We demonstrate complete follicular development and recovery of endocrine function after cryopreservation and autotransplantation of small ovarian fragments in bilaterally ovariectomized goats without administration of hormones. Previous experiments had demonstrated that ovarian tissue of large animals such as goats can be cryopreserved more efficiently when divided into small fragments (2). After freezing-thawing, viable early stage follicles (primordial up to and including secondary) could be identified, and only primordial follicles survived to transplantation. Between 2 and 3 months after transplantation, we observed antral and preovulatory follicles and corpora lutea in ovarian-like structures. This indicates the ability of cryopreserved primordial follicles to develop completely until the ovulatory stage and to ovulate. In addition, our findings were accompanied by increased levels of E_2. Recovery of endocrine function has also been reported in mice after transplantation of cryopreserved ovarian tissue. However, in most studies the whole ovary was grafted (6), sometimes in hemiovariectomized recipients (7), instead of only a minimal part of the ovary in completely ovariectomized recipients as in the present study. Furthermore, the current study demonstrates that gonadal function can be recovered even when only a small fraction (approximately 30%) of one ovary is transplanted. Cyclic estrous behavior was observed in all animals that had received transplants but was not always accompanied by ovulation and formation of corpora lutea. Anovulation in these animals was not caused by an increase in T (8) but more likely due to too-low E_2 levels, which apparently were sufficient to stimulate estrous behavior but insufficient to support finalization of folliculogenesis and subsequent ovulation. Approximately 50% of early stage follicles survived in transplants, and follicular density was decreased significantly. This was observed not only in frozen-thawed transplants but also in fresh transplants, indicating that it is not the cryopreservation per se but the surgical procedure that damages early stage follicles. Duration of folliculogenesis, that is, the development of a primordial follicle up to the preovulatory stage, was determined by the first rise in the serum E_2 level after transplantation, and appeared after approximately 70 days, similar to that reported for adult women (9). Because, at early developmental stages, mean diameters of goat oocytes and follicles are also more similar to those of humans than to those of other livestock animals (10), the goat is an excellent model to study ovarian function. The current study demonstrates the recovery of ovarian function even if only small cortical biopsy specimens after cryopreservation are transplanted. Further studies in ovarian tissue cryopreservation and transplantation with use of goats as animal models will help to develop safe procedures before clinical application.

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