In vivo characterization of metabolic activity and oxidative stress in grafted human ovarian tissue using microdialysis

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Objective: To characterize oxidative stress and metabolic activity in xenografted human ovarian tissue using microdialysis.
Design: Prospective experimental study.
Setting: Gynecology research unit at a university hospital.
Patient(s): Cryopreserved ovarian cortex from five women 27–35 years of age.
Intervention(s): Frozen-thawed human ovarian tissue fragments were xenografted to the back muscle of ten nude mice. Before grafting, a microdialysis probe was placed inside each fragment.
Main Outcome Measure(s): Daily reactive oxygen species (ROS), lactate, and glucose levels were collected by means of microdialysis. Follicle loss (hematoxylin and eosin), murine and human vascularization, and vessel stability (CD31, von Willebrand factor, and α-smooth muscle actin triple immunofluorescence) were analyzed on post-grafting days 10 and 21.
Result(s): Lactate levels were significantly higher than glucose levels until day 10, after which time the lactate-glucose ratio stabilized at ~1:1. Regarding ROS generation, there were two peaks on post-grafting days 10 and 17. Total vascularization increased significantly up to day 10 and remained similar up to day 21. However, murine vessel area and stabilization significantly increased up to day 21. Major follicle loss occurred in the first 10 days after transplantation.
Conclusion(s): Our data validated microdialysis as a tool to characterize metabolic behavior and oxidative stress in grafted ovarian tissue. Three different post-grafting periods were identified according to the metabolic activity of grafted tissue, showing a long progression from anaerobic to aerobic metabolism and a protracted period of ROS generation. Oxidative stress was observed relatively late, after the most critical period of follicle loss, and lasted until the tissue vasculature stabilized. (Fertil Steril® 2018;■: ■–■. ©2018 by American Society for Reproductive Medicine.)
Key Words: Ovarian tissue transplantation, oxidative stress, ROS, metabolism, microdialysis

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Advances in cancer treatment in recent decades have increased life expectancy in girls and young women, resulting in growing numbers of long-term cancer survivors who may experience premature ovarian failure and infertility due to gonadotoxic therapy (1). Among fertility preservation techniques, ovarian tissue cryopreservation and transplantation is the only approach available for prepubertal girls and patients who require immediate cancer therapy (2). Ovarian cortex is transplanted without vascular anastomosis and undergoes a period of ischemia and hypoxia before revascularization, depending on vessel growth and invasion (3). This leads to follicle loss of 50%–90% (4, 5). In 2009, van Eyck et al. used electron paramagnetic resonance (EPR) oximetry (6) to prove that hypoxia lasts for 3–5 days and

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that partial pressure of oxygen stabilizes ~10 days after transplantation in a human ovarian tissue xenografting model.

Several attempts have been made to limit ischemic and hypoxic damage in grafted ovarian tissue. Because oxygen free radicals are one of the main damaging components in the ischemia-reperfusion process (7), antioxidants have been widely used to enhance follicle survival and graft viability after transplantation (3). Various antioxidants, such as vitamin E (8) and melatonin (9) in xenografting models with human ovarian tissue, and erythropoietin (10, 11), L-carnitine (12), and N-acetylcysteine (13) in autografting models with canine and murine ovarian tissue, have been shown to yield some improvement in follicle apoptosis and lipid peroxidation. However, no meaningful benefits of antioxidant use were demonstrated in grafted human ovarian tissue (3).

Oxidative stress is usually studied indirectly in grafted ovarian tissue, through evaluation of scavenger expression and lipid peroxidation (8–13), because direct quantification of reactive oxygen species (ROS) is not always technically possible owing to ROS instability. However, hydrogen peroxide (H_2O_2) can be detected with a sensitive and specific fluorogenic dye, such as Amplex Ultrared (14, 15).

Our present objective was to characterize metabolic activity and ROS production in human ovarian tissue after transplantation with the use of microdialysis for collection of lactate as an anaerobic metabolism marker, glucose as a blood flow marker (16), and H_2O_2 concentrations as oxidative stress markers (17, 18). To better understand the metabolic behavior of grafted ovarian tissue and oxidative stress, we applied microdialysis for continuous sampling of metabolites present in the extracellular space, which acts like a capillary vessel, allowing their passage in the dialysate by simple diffusion before harvesting and subsequent analysis (19, 20).

MATERIALS AND METHODS

Experimental Design

Ten nude mice (Swiss nu/nu) were each grafted with a fragment of frozen-thawed human ovarian tissue taken from five different patients. A microdialysis probe was placed inside each piece before grafting to the back muscle. Ovarian tissue from each patient was divided equally among three groups: one piece for nongrafted control and the other two for xenografting. Biopsies from different patients varied in size, so thawed samples were cut into equal 6 × 4 × 2–mm fragments and evenly distributed among the groups. Microdialysis was performed daily in each grafted mouse. Hoechst 33342 was injected before killing them to reveal ovarian graft perfusion. The mice were killed on days 10 (n = 5) and 21 (n = 5) after transplantation, and the grafted tissue was fixed for histology and immunofluorescence (Fig. 1).

Ovarian Tissue Collection and Thawing Procedure

Use of human ovarian tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain (2012/23MAR/125). Ovarian tissue biopsies from five women (ages 27–35 years) were taken from our tissue bank after obtaining written informed consents. All five women had undergone surgery for non-ovarian pathologies. Freezing of ovarian tissue was achieved with the use of the slow-freezing protocol. Briefly, strips of tissue were suspended in cryoprotective solution consisting of Leibovitz-15 medium (Gibco) supplemented with 4 mg/mL human serum albumin (Sanquin) and 1.5 mol/L dimethylsulfoxide (Sigma) at 4°C and transferred to 2 mL cryovials (Simport) containing 0.8 mL cryoprotectant. The cryovials were cooled in a programmable freezer (Freezer Control CL-8800i; Cryologic) and transferred to liquid nitrogen (−196°C) for storage (21).

Cryogenic vials containing the ovarian tissue fragments were thawed at room temperature for 2 minutes, immersed in a water bath at 37°C for another 2 minutes, then washed three times in fresh HEPES-MEM (Gibco) to remove the cryoprotectant.

Surgical Procedure

Animal welfare guidelines were strictly followed, and the protocol was approved by the Committee on Animal Research of the Université Catholique de Louvain (2014/UCL/M.D./007). Ten nude female mice (Crl:NU-Foxn1nu, Swiss nu/nu; Charles River Laboratories) 6–9 weeks old were used for this study. This specific animal model was chosen because these are athymic outbred immunodeficient (no T cells) mice, ideal for xenografting experiments. Satisfactory housing and breeding conditions were ensured, as previously described (5). Isoflurane (Zoetis) provided anesthesia (3% for induction and 1.5% for maintenance), with continuous control of air-breathing movements. The back muscle (22) was exposed by means of an L-shaped incision. The ovarian tissue was pierced along its length with a 23-G needle to allow insertion of the probe. The ovarian tissue and probe mesh were then both fixed to the same muscle surface with the use of stitches (6/0 Prolene; Ethicon; Johnson & Johnson International) after scratching the muscle aponeurosis (23). Two small holes were made caudal to the incision to create inlet and outlet tube passages for continuous measurement (Fig. 1). The skin incision was then closed with the use of 4/0 Prolene (Ethicon).

Microdialysis Set-Up

A microdialysis probe is composed of a semipermeable membrane that is inserted into target tissue and perfused with a constant flow (perfusate). The perfusate flows through the probe, and the outflowing solution (dialysate) is collected for analysis. Analyte recovery from the dialysate depends on membrane pore size and is inversely proportional to flow rate and directly proportional to membrane length (19). Analyte concentrations in the dialysate correlate directly with amounts found in extracellular fluid surrounding the microdialysis probe, when membrane porosity, probe length, and perfusion rate are constant parameters.

For this experiment, serial custom-made probes were used, consisting of a 4-mm semipermeable membrane of regenerated cellulose, pore size equivalent to 18 kDa (Brainlink) and a mesh on the probe body to be stitched to the tissue