Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting

Anne-Sophie Van Eyck, M.D.,[a] *Bénédicte Francine Jordan, Pharm.D., Ph.D.,*[b] *Bernard Gallez, Pharm.D., Ph.D.,*[b] *Jean-François Heilier, Pharm.D., Ph.D.,*[c] *Anne Van Langendonckt, M.Sc., Ph.D.,*[a] *and Jacques Donnez, M.D., Ph.D.*[a]

[a] Department of Gynecology, Faculty of Medicine, Université catholique de Louvain, Brussels, Belgium; [b] Laboratory of Biomedical Magnetic Resonance, Université catholique de Louvain, Brussels, Belgium; and [c] Industrial Toxicology and Occupational Medicine Unit, Faculty of Medicine, Université catholique de Louvain, Brussels, Belgium

Objective: To develop electron paramagnetic resonance (EPR) oximetry as a tool to characterize the oxygen environment in human ovarian xenografts in the early postgrafting period.
Design: Prospective experimental study.
Setting: Gynecology research unit in a university hospital.
Patient(s): Biopsies were obtained from 6 women aged 22–35 years.
Intervention(s): Frozen–thawed human ovarian tissue fragments were grafted to an intraperitoneal site in nude mice. Before grafting, lithium phthalocyanine, an oxygen reporter, was implanted inside the fragments.
Main Outcome Measure(s): To monitor partial pressure of oxygen (pO_2) by EPR on postgrafting days 3, 5, 7, 10, 14, 17, and 21 and validate the technique by histologic assessment.
Result(s): A period of hypoxia was identified before day 5, followed by gradual but significant oxygenation over the next 5 days, suggesting an active process of graft revascularization. Reoxygenation kinetics in human ovarian xenotransplants were quantified.
Conclusion(s): Our data validated the EPR oximetry technique as a tool to monitor pO_2 in ovarian grafting. The critical early period of hypoxia was identified, and the first steps of reoxygenation were characterized. In the future, our model may be used to evaluate new freezing and grafting protocols with the aim of reducing potential cryoinjury and initial ischemia–reperfusion damage. (Fertil Steril® 2009;92:374–81. ©2009 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, human ovarian xenotransplantation, EPR oximetry, angiogenesis, hypoxia

In recent years improvements in oncologic treatment regimens have led to an increase in life expectancy for young female cancer patients. Unfortunately, aggressive chemotherapy, ionizing radiotherapy, and bone marrow transplantation can severely affect the ovarian follicular reserve and subsequently lead to loss of fertility and premature menopause. Ovarian tissue cryopreservation before the initiation of gonadotoxic treatment, with the aim of reimplanting the frozen–thawed ovarian tissue once the patient has recovered, is an option to restore endocrine and reproductive function (1). Since the first report of a live birth after autografting of frozen–thawed ovarian tissue was published by our team (2), three more live births have been achieved by this approach (3–5).

Although these results are very encouraging, this technique remains experimental, and further research is required. Indeed, experimental studies have demonstrated considerable loss of primordial follicles in cryopreserved ovarian tissue after transplantation, estimated to be 50%–65% by some investigators (6, 7) and >90% by others (8, 9). This phenomenon could be explained by the fact that ovarian cortical pieces are grafted without vascular anastomosis. Implants are thus exposed to ischemic damage and oxidative stress during the early posttransplantation period until they become revascularized. Lipid peroxidation and oxygen-deprived free radicals seem to be involved in this ischemia–reperfusion injury, as suggested by the improvement in follicular survival rates from 45% to 72% in case of antioxidant administration to the host (10).

In rodents, Israely et al. (11) showed that the avascular period was associated with follicular damage, as illustrated by the apoptotic death of primordial follicles and perivascular endothelial cells. When partially perfused, ischemic areas were found to co-localize with degenerated/necrotic follicles, whereas perfused areas contained intact follicles. They also demonstrated that shortening the hypoxic period resulted in

Received January 16, 2008; revised April 30, 2008; accepted May 1, 2008; published online August 11, 2008.
Supported by grants from the Fonds National de la Recherche Scientifique de Belgique (Research Fellow, grant no. 7.4547.06), the Fondation St. Luc, the Foundation Against Cancer, and donations from Baron Frère and Vicomte Philippe de Spoelberch.
Presented at the Congress "Oocytes, Ovary and Transplantation: New Discoveries Applied to Fertility Preservation," September 27–29, 2007, Boston, Massachusetts.
A.-S.V. has nothing to disclose. B.F.J. has nothing to disclose. B.G. has nothing to disclose. J.-F.H. has nothing to disclose. A.V. has nothing to disclose. J.D. has nothing to disclose.
Reprint requests: Jacques Donnez, M.D., Department of Gynecology, Université catholique de Louvain, Cliniques Universitaires St. Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium (FAX: 32-2-764.95.02; E-mail: donnez@gyne.ucl.ac.be).

0015-0282/09/$36.00
doi:10.1016/j.fertnstert.2008.05.012

improved survival of primordial follicles and restoration of follicular growth.

Because the duration of ischemia and the rate of apoptosis of follicles seem to be correlated (12), a better understanding of the initial ischemic processes leading to revascularization of human ovarian grafted tissue is crucial, but effective methods to analyze these first steps are lacking.

The aim of our study was to set up an electron paramagnetic resonance (EPR) protocol to characterize the oxygen environment after ovarian tissue xenotransplantation and identify the initial hypoxic and reoxygenation periods after human ovarian transplantation in the early postgrafting phase.

MATERIALS AND METHODS

EPR Oximetry

The EPR oximetry technique enables sensitive, noninvasive and repeated measurement of partial pressure of oxygen (pO_2) in vivo, notably in tumoral models, to characterize the tumor microenvironment (13). Based on the paramagnetic properties of molecular oxygen, which modifies the EPR spectra by altering the relaxation rates of paramagnetic species, the technique allows continuous monitoring of oxygenation in tissues with an accuracy of <1 mm Hg (14).

The magnitude of the effect is related to the amount of oxygen that is present in the environment of the paramagnetic materials (14). Lithium phthalocyanine (LiPc) was used as an oxygen-sensitive paramagnetic probe, which was inserted into the ovarian fragments before grafting. The line width of LiPc is a linear function of pO_2 and is independent of local metabolic processes, other paramagnetic species, and pH.

Experimental Design

A total of 24 mice were intraperitoneally grafted with frozen–thawed human ovarian fragments obtained from 6 different patients. Eighteen nude mice were grafted with ovarian fragments into which LiPc had been implanted as an oxygen reporter. Six control mice were grafted without LiPc. These 6 control mice were included for histologic comparison with the lithium-grafted mice on day 21.

Levels of pO_2 were monitored in vivo by EPR on postgrafting days 3, 5, 7, 10, 14, 17, and 21 ($n = 18$). As described by Gallez and Mader (15), the stability of LiPc responsiveness to pO_2 was confirmed by modification of the line width, following a clear change in pO_2 values (15). Carbogen breathing challenges (positive controls) were applied on days 3 and 21, and hypoxic conditions (negative controls) were obtained by pO_2 measurement after the mice were killed on day 21.

Collection of Ovarian Tissue

The use of human tissue for this study was approved by the Institutional Review Board of the Université catholique de Louvain. Ovarian biopsies were taken from 6 women (aged 22–35 years) after written informed consent was obtained. They were all undergoing laparoscopic surgery for non-ovarian benign gynecologic disorders, such as tubal ligation, peritoneal endometriosis, or myomas. One biopsy of 8–10 mm $\times$ 4 mm was taken per patient, from which four fragments of 2–4 mm were obtained.

Freezing and Thawing Procedure

Freezing of ovarian tissue was undertaken according to the protocol described by Gosden et al. (16). Biopsy samples were immediately transferred from the operating room to the research laboratory in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered modified Eagle's medium (HEPES-MEM) (GIBCO, Glasgow, Scotland) on ice. The medullar part was removed from the ovarian biopsies using surgical scissors. Ovarian tissue was dissected into strips of 2×4 mm. These fragments were suspended in cryoprotective medium (HEPES-MEM supplemented with 4 mg/mL of human serum albumin [Red Cross, Brussels, Belgium] and 1.5 mmol dimethyl sulfoxide [DMSO; Sigma, St. Louis, MO]) in cryogenic vials (Simport, Quebec, QC, Canada). The cryotubes were cooled in a programmable freezer (Kryo 10, Series III; Planer, Sunbury-on-Thames, United Kingdom) using the following program: [1] cooled from 0°C to −8°C at −2°C/min, [2] seeded manually, [3] cooled to −40°C at −0.3°C/min, [4] cooled to −150°C at −30°C/min, and [5] transferred to liquid nitrogen (−196°C) for storage. The time interval between biopsy and the start of freezing was approximately 20 minutes in all cases. The cryogenic vials containing the fragments were thawed at room temperature for 2 minutes and immersed in a water bath at 37°C for another 2 minutes. The ovarian tissue was washed three times with fresh HEPES-MEM medium to remove the cryoprotectant. The interval between thawing and transplantation was approximately 30 minutes.

Implantation of LiPc into Ovarian Tissue and Transplantation

Twenty-four nude (NMRI nu/nu) 7–10-week-old female mice (Janvier, Le Genest St. Isle, France) were operated on. The mice were bred as previously described (7).

Intraperitoneal injection of ketamine (75 mg/kg Anesketin; Eurovet, Heusden-Zolder, Belgium) and medetomidine (1 mg/kg Domitor; Pfizer, Cambridge, MA) was administered for anesthesia, and buprenorphine (0.1 mg/kg Temgesic; Schering Plough, Kenilworth, NJ) for analgesia. After surgery, anesthesia was reversed by injection of atipamezole (1 mg/kg Antisedan; Pfizer).

Eighteen mice were each grafted with one ovarian fragment containing LiPc (three fragments obtained from each of the 6 patients) for pO_2 monitoring by EPR.

Before implantation, the LiPc crystals were sterilized by dry heating, as described by Dinguizli et al. (17). The LiPc was then implanted inside the ovarian fragments using a 22-gauge injection needle. Regarding the high spin density

of LiPc, the signal-to-noise ratio of the spectra was previously shown to be adequate with small amounts of crystals (18). The needle tip was thus filled with four LiPc crystals with a maximum width of 400 μm.

The cortical fragments were placed into fresh HEPES-MEM at 4°C until transplantation.

A horizontal incision was made to fix the ovarian fragments to the lower third of the parietal peritoneum with Prolene 7/0. The abdominal wall and skin were then closed with 7/0 Prolene.

In the same manner, 6 control mice were grafted with fragments without lithium (one fragment from each patient) for histologic comparison on day 21.

EPR Measurements

The EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Typical spectrometer parameters were modulation amplitude less than one third of the peak-to-peak line width, incident microwave power of 50 μW, and 10 kHz modulation frequency.

For pO_2 measurements, the mice were anesthetized using isoflurane (3% for induction, 2% for maintenance) with strictly controlled air-breathing movements. Body temperature was maintained by use of a heated water blanket. The mice were positioned in the magnet so that the graft site was directly under the extended loop resonator.

The EPR line widths were converted to pO_2 by means of a calibration curve determined for the LiPc crystals used in this study. The pO_2 was calculated from the following equation: pO2 (mm Hg) = ΔB (mT) − 0.0031/0.0005.

The recorded spectrum revealed the mean pO_2 value over the surface of the crystals.

The pO_2 measurements could not be taken on the day of surgery because a certain amount of time is required for LiPc crystals to equilibrate with tissue before pO_2 measurements can be started (19). The presence of residual air in the abdominal cavity due to surgery, or air around the crystals due to the injection procedure, could falsely increase pO_2 levels. Indeed, Liu et al. (18) demonstrated that calibrations of line widths of the EPR spectra of LiPc to pO_2 under various conditions, including the gas phase and tissue, were similar, showing a broadening effect of oxygen independent of the surrounding medium. Preliminary experiments (n = 3) performed from days 1 to 3 revealed stable values (day 1: 9.1 mm Hg ± 4.2 mm Hg; day 2: 7.8 mm Hg ± 3.5; day 3: 10.5 mm Hg ± 4.6 mm Hg), suggesting an absence of reoxygenation during this period. Monitoring of pO_2 values was therefore initiated on day 3.

After ambient air measurements, positive and negative controls were applied to confirm oxygen sensitivity of the paramagnetic probes for each mouse. Carbogen breathing challenges (95% O_2, 5% CO_2) were applied as positive controls. As negative controls, pO_2 levels were measured reproducibly 5 minutes after the mice were killed by cervical dislocation. In the absence of oxygenated blood circulation, postmortem values were considered to be indicative of a hypoxic state.

Validation by Histologic Analysis

In both groups (ovarian tissue grafted with or without LiPc), recovered tissue was fixed in 4% formaldehyde, embedded in paraffin, and serially sectioned (5-μm-thick sections). Slides were stained with hematoxylin-eosin (Merck, Darmstadt, Germany) for histologic evaluation. Twenty serial sections were examined under a light microscope at ×200 magnification to evaluate follicular density. Only follicles with a visible nucleus were counted to avoid counting follicles twice. The percentage of atretic follicles was assessed; pyknotic granulosa cells, eosinophilia of the ooplasm, and contraction and clumping of chromatin were all considered to be signs of atresia (20). On the basis of classic histologic criteria, fibrotic and necrotic areas in grafts were evaluated on two slides. Fields were digitized using a Leica DFC320 camera and IM50 program (Leica, Wetzlar, Germany). ImageJ, an image-processing and analysis program developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/) was used to delimit surfaces.

The vascular network of mouse and human origin was evaluated by CD-34 immunolabeling, staining endothelial cell membranes. First, primary CD-34 rat monoclonal anti-mouse antibody (1:100 dilution, clone MEC 14.7, HM 1015; Hycult Biotechnology, Uden, The Netherlands) was used, followed by biotinylated rabbit anti-rat IgG (1:100 dilution, BA 4001; Vector Laboratories, Burlingame, CA) and streptavidin–horseradish peroxidase (1:1000 dilution, P0397; DakoCytomation, Carpinteria, CA). Thereafter, CD-34 mouse monoclonal anti-human antibody (1:8000 dilution, clone QBEnd/10, CM084B; Biocare Medical, Concord, CA) and EnVision+ System anti-mouse horseradish peroxidase–labeled polymer (K 4001; DakoCytomation) were applied. Diaminobenzidine (Dako) was used as a chromogen, and nuclei were counterstained with hematoxylin. Positive and negative controls were included in the series. Vessel sections were considered positive when at least one endothelial cell was stained. The results were reported as vascular density and vascular surface area evaluated by morphometry, as described above.

Statistical Analysis

Commercial software (SAS/STAT 8; SAS Institute, Cary, NC) was used for statistical analyses. Comparisons between pO_2 monitoring times and graft conditions (with or without LiPc, n = 6 patients) were performed by the paired Student's *t*-test or Fisher's exact test, as appropriate.

Reoxygenation speed (individual slope of linear regression from day 3 to 10) was determined with Prism (GraphPad

Software, San Diego, CA). Comparison of mean reoxygenation slope to null hypothesis (reoxygenation slope: 0 mm Hg/d) was performed using Student's *t*-test. A *P* value of $<.05$ was considered statistically significant.

RESULTS

Oxygen Sensitivity of LiPc

Lithium phthalocyanine crystals may lose their responsiveness to pO_2 over time, depending on the implantation site, and no information is available on LiPc stability in ovarian tissue. For this reason, positive and negative controls were included to confirm the responsiveness of the paramagnetic probes to pO_2 during the experiment (15).

The pO_2 was monitored in all grafted mice (n = 18) but only analyzed in 14 mice. Indeed, 3 mice were excluded because negative controls on day 21 after killing were not hypoxic, suggesting progressive loss of responsiveness of LiPc to pO_2. One mouse presented with postoperative complications, which led to exclusion due to modification of the postgrafting environment.

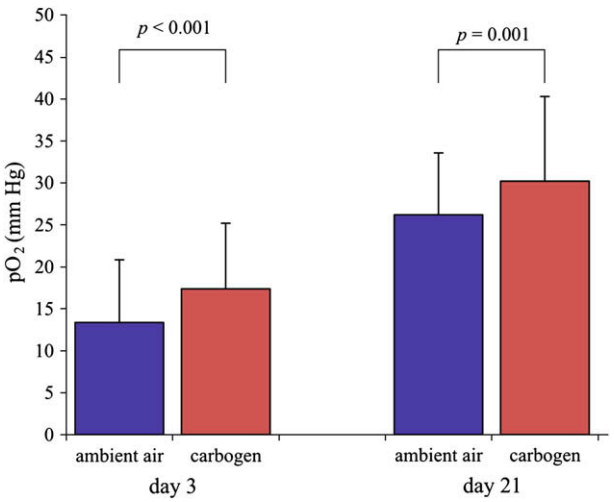
The results of the positive and negative controls confirmed the responsiveness of LiPc to pO_2, following clear changes in pO_2 conditions (Fig. 1).

On day 3 the pO_2 level obtained after the carbogen breathing challenge was significantly higher than under ambient air conditions (mean ± SD: 13.3 ± 7.4 mm Hg and 17.3 ± 7.7 mm Hg [$P<.001$] for day-3 room conditions and carbogen breathing, respectively).

On day 21 the pO_2 level increased after the carbogen breathing challenge (26.2 ± 7.3 mm Hg and 30.2 ± 11.1 mm Hg [$P<.001$] for day-21 room conditions and carbogen breathing, respectively). After killing, the pO_2 level significantly decreased (26.2 ± 7.3 mm Hg and 10.5 ± 1.9 mm Hg [$P<.001$] for day-21 room conditions and postmortem measurements, respectively).

FIGURE 1

Responsiveness of LiPc to pO_2: positive controls (n = 14 mice). Positive controls (carbogen-breathing challenges) carried out on days 3 and 21 showed significantly higher levels than ambient air conditions on the same days, which confirmed the responsiveness of LiPc to pO_2, following clear changes in pO_2 conditions (mean ± SD, paired Student's *t*-test).



Van Eyck. EPR to assess ovarian graft oxygenation. Fertil Steril 2009.

pO_2 Monitoring

Values obtained on day 3 were hypoxic, similar to postmortem measurements (13.3 ± 7.4 mm Hg and 10.5 ± 1.9 mm Hg on day 3 and day 21 postmortem, respectively). From day 5, significant reoxygenation was observed (day 3: 13.3 ± 7.4 mm Hg; day 5: 22.3 ± 11.6 mm Hg; $P<.0001$).

Reoxygenation speed from day 3 to day 10 was identified for each patient graft and was reproducible between patients. The pO_2 level significantly increased from day 5 to day 10 (slope = 2.4 ± 0.4 mm Hg/d; $P<.001$). The highest level obtained on day 10 was still maintained on day 14 (31.9 ± 11.05 mm Hg and 31.1 ± 7.02 mm Hg on day 10 and day 14, respectively) (Fig. 2).

From day 17, pO_2 values stabilized (day 17: 24.8 ± 7.2 mm Hg; day 21: 26.2 ± 7.3 mm Hg) and remained higher than the initial hypoxic value observed on day 3 (day 3: 13.3 ± 7.4 mm Hg; day 21: 26.2 ± 7.3 mm Hg; $P<.001$).

Histologic Analysis: Inertness of LiPc on Ovarian Structure

In all cases, histologic examination demonstrated the presence of LiPc crystals inside the ovarian grafts (Fig. 3).

Lithium phthalocyanine did not interfere with ovarian structure, as evidenced by histologic comparison between the lithium-grafted group and the grafted control group, summarized in Table 1. No statistical difference was observed between the two groups with respect to follicular density, atretic follicle percentages, or fibrotic areas. Vascular networks (area and density) revealed by CD-34 double immunolabeling were similar in both groups, suggesting that LiPc implanted inside the ovarian fragments did not alter the revascularization process.

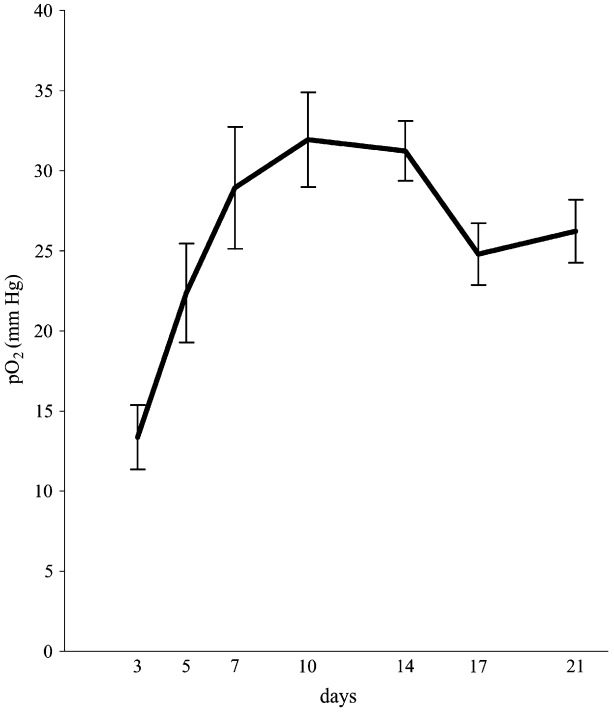
Regarding necrosis, no necrotic areas were observed in the control group. In the lithium-grafted group, a low rate of necrosis was observed near the LiPc crystals (percentage of necrotic surface area ± SE: 1.005% ± 1.465%), which could have been due to injury caused by the insertion of the LiPc into the fragments.

DISCUSSION

Cryopreservation and transplantation of ovarian fragments is a promising approach to preserve fertility after gonadotoxic treatments (1). Loss of primordial follicles and follicular activation, illustrated by the increase in the proportion of

FIGURE 2

Monitoring of pO_2 values (mean ± SEM, n = 6 patients). The pO_2 levels significantly increased from day 5 to day 10. Reoxygenation kinetics were characterized by reoxygenation speed (slope = 2.4 ± 0.4 mm Hg/d; $P < .001$), specific to our graft model. The highest level obtained on day 10 was still maintained on day 14. From day 17, pO_2 values were stable until day 21 and remained higher than the value observed on day 3.



Van Eyck. EPR to assess ovarian graft oxygenation. Fertil Steril 2009.

growing follicles observed in experimental models, could be due to the period of hypoxia following avascular grafting procedures (9, 21, 22). Indeed, grafts are exposed to ischemic tissue damage during the early posttransplantation period until the fragments become revascularized.

To our knowledge, no information is available on the hypoxic period and the first steps of revascularization in human ovarian xenografts. Electron paramagnetic resonance oximetry is the only technique designed for continuous in vivo measurement of local pO_2 levels, without altering local oxygen concentrations. Electron paramagnetic resonance allows repeated pO_2 measurements from the same site over long periods (14). In our study, EPR oximetry was applied for the first time to evaluate human ovarian tissue reoxygenation after xenografting. This technique enabled us to identify the critical early period of hypoxia, to quantify its extent, and to gather essential information on the first steps of reoxygenation after avascular ovarian fragment grafting.

Technical Aspects

Several technical aspects needed to be considered to apply the technique to our human ovarian xenografting model.

The intraperitoneal site, used by our team in both xenotransplantation models (7) and clinical application (2), was chosen. Emitted signals could be detected by the EPR loop resonator, whose detection is limited to a depth of 10 mm.

To reduce the physiologic movements of the animals (e.g., breathing movements and intestinal peristalsis) that could decrease the spectrum signal/noise ratio, the tissue fragments were fixed to the lower third of the parietal peritoneum. The graft was thus relatively isolated from intestinal structures. Moreover, the anesthesia was strictly controlled to maintain regular and superficial breathing movements.

As routinely practiced, investigation of the stability of the response to pO_2 in experiments (positive and negative controls) and histologic analysis after killing were performed.

No information is currently available on LiPc stability when implanted inside ovarian tissue. According to some investigators, LiPc crystals may lose their response to pO_2 over time depending on implantation site, as described in skeletal muscle and oral mucosa (18–23). The time span of LiPc usefulness in vivo therefore depends on the type of tissue environment (18). With ovarian fragments grafted to an intraperitoneal site, LiPc responsiveness to pO_2 was maintained for 21 days.

However, 3 mice lost responsiveness to O_2 during pO_2 monitoring, as proved by the absence of hypoxic values after killing on day 21. Histologic analysis of these 3 ovarian grafts (data not shown) revealed multinucleated giant cells accumulated around the LiPc crystals. Even with the use of immunodeficient mice, partial rejection of human grafts can occur, as evidenced by signs of an inflammatory response. In normal conditions, O_2 access occurs through small opening channels in the crystals (18). Loss of responsiveness could be due to deposition of material in and around the LiPc crystals, which impedes oxygen access to the paramagnetic center. Erjavec et al. (23) suggested that a prolonged inflammatory response could shorten the period of sensitivity of paramagnetic probes to pO_2.

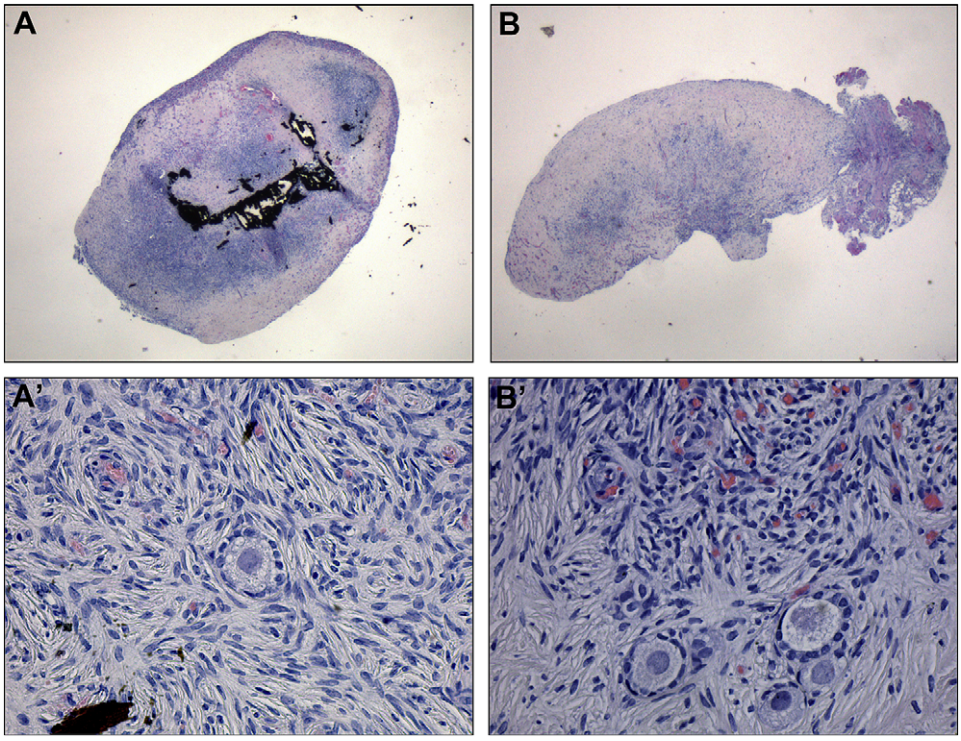
Liu et al. (18) described the physicochemical stability of LiPc, including resistance to oxidation, reduction, and pH extremes. Histologic studies of tissues containing implanted LiPc, such as muscle, brain, and heart, confirmed these findings (24). In ovarian grafts, LiPc crystals did not seem to interfere with the histologic parameters analyzed, as previously observed in other tissues.

Contribution of the Technique

Using this new EPR protocol, we were able to identify a state of improved oxygenation from day 5 onward, with higher pO_2 levels than the initial hypoxic state observed before day 5.

FIGURE 3

(**A**) Ovarian fragment grafted with LiPc (hematoxylin-eosin stain, original magnification ×12.5). (**A'**) Healthy follicles near the LiPc in normal stroma (hematoxylin-eosin stain, original magnification ×200). (**B**) Ovarian fragment grafted without LiPc (hematoxylin-eosin stain, original magnification ×12.5). (**B'**) Healthy follicles in partially fibrotic stroma (original magnification ×200).



Van Eyck. EPR to assess ovarian graft oxygenation. Fertil Steril 2009.

During this initial phase, follicles are exposed to pO_2 levels similar to those observed after death in the absence of blood oxygenation, leading to ischemic injury. Tatone et al. (25) recently highlighted the role of the microenvironment in oocyte aging and suggested that an environment compromised by reactive oxygen species and a reduced oxygen supply may be involved in oocyte senescence.

Lack of O_2 may alter ovarian tissue morphophysiology on different levels. First, hypoxia may induce necrosis or activation of apoptotic pathways, which could explain ovarian tissue impairments, such as massive follicular loss (6–9). Furthermore, premature follicular cell activation, contributing to premature follicular depletion of the graft, may be due to granulosa cell hypoxia-induced proliferation through

TABLE 1

Histological parameters comparing ovarian fragments grafted with and without LiPc.

Parameter	Without LiPc	With LiPc	*P* value
Follicular density (no. of follicles/mm^3), mean ± SD	44.5 ± 24.6	36.7 ± 24.3	.22[a]
Atretic follicles (atretic/total no. of follicles)	6/112	4/100	.75[b]
Fibrotic area (%), mean ± SD	56.1 ± 22.1	57.1 ± 12.0	.91[a]
Vascular area[c] (%), mean ± SD	3.3 ± 4.6	4.1 ± 4.7	.63[a]
No. of capillaries per mm^2[c]	153.1 ± 171.9	165.3 ± 132.1	.77[a]

Note: No statistical difference was observed between the groups for the parameters analyzed.
[a] Paired *t*-test.
[b] Fischer's exact test.
[c] Vascular area and density revealed by CD-34 double immunolabeling.

Van Eyck. EPR to assess ovarian graft oxygenation. Fertil Steril 2009.

hypoxia-inducible factor-1 (HIF-1) transduction pathways (26, 27). Such events have been observed in rodent ovarian tissue within this time frame (9, 11, 21, 22, 28). Activated at low oxygen tension, HIF-1 seems to control transcriptional upregulation of vascular endothelial growth factor, allowing cellular adaptation to hypoxia (29). Yang et al. (28) recently identified an initial increase in mRNA and protein expression of vascular endothelial growth factor 2 to 7 days after transplantation.

The process of reoxygenation in ovarian fragments seems to be faster than that observed in other tissues. Indeed, follow-up of tissue oxygenation in ischemia–reperfusion or wound angiogenesis models reveals that hypoxic values persist more than 7 days after femoral artery occlusion in the absence of initial collateral vascularization and more than 10 days in case of wound-healing processes (14–30). The increase in pO_2 observed on day 5 in our model strongly suggests an intense and active process of graft revascularization in ovarian tissue, leading to subsequently improved graft oxygenation. These results are supported by observations made after transplantation of cryopreserved human ovarian tissue in the chorio-allantoid membrane model, showing invasion of the ovarian stroma by avian blood vessel from day 3, becoming more apparent on days 4 and 5 (31). In rodents, a short avascular period followed by prompt and massive revascularization within 48 hours was reported by Dissen et al. (32), whereas Israely et al. (33) showed that grafts remained avascular on days 1–3 after transplantation but showed functional vessels on day 7.

Thanks to the unique characteristics of the EPR technique, allowing repeated measurements to be taken from the same site over time, the reoxygenation kinetics could be quantified in our grafting model. Reoxygenation speed was reproducible between patients. The maximum pO_2 level obtained on day 10 was consistent with other observations made in our laboratory showing a progressive increase in the perfusion area of the fragment, initially peripheral, leading to complete fragment perfusion by day 10 (unpublished data).

Furthermore, pO_2 values were stable from day 17 to 21, suggesting stabilization of the graft revascularization process. Indeed, pO_2 levels observed after 21 days suggest that the graft was just as vascularized as other intra-abdominal structures, such as the liver, showing a median value of 23.4 mm Hg (34).

Damage observed after ovarian xenotransplantation suggests that further research is needed to improve graft conditions. The EPR technique may be used to assess whether new grafting protocols could shorten the period of hypoxia and increase reoxygenation speed to reduce exposure time of the graft to ischemia–reperfusion damage. Indeed, a number of investigators have tried to improve angiogenesis and minimize hypoxic tissue damage by adding antioxidant agents in animal ovarian tissue transplantation models (10–12). Moreover, it was recently suggested that cryopreservation procedures could be implicated in ovarian tissue damage, underestimated by simple histologic and ultrastructural analyses immediately after cryopreservation (9). Electron paramagnetic resonance oximetry could therefore be an interesting technique to evaluate the effect of new freezing protocols on the survival of ovarian tissue by evaluation of graft recovery.

In conclusion, we investigated and illustrated the effectiveness of a new methodology using EPR oximetry to evaluate the oxygen environment in human ovarian xenotransplantation. A period of hypoxia was identified before day 5, followed by gradual reoxygenation.

Further studies are required to evaluate the biological impact of oxygenation levels on various markers, including apoptosis and angiogenesis, at the main time points studied.

In the future our model may be used to assess new freezing and grafting protocols with the aim of reducing potential cryoinjury and initial ischemia–reperfusion damage.

Acknowledgments: The authors thank Mira Hryniuk and Christiani Andrade Amorim for reviewing the manuscript; Professor H.M. Swartz (Dartmouth Medical School, Hanover, NH) for the generous gift of lithium phthalocyanine crystals; and Dolores Gonzalez for her technical assistance.

REFERENCES

1. Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update 2006;12:519–5.
2. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;364:1405–10.
3. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N Engl J Med 2005;353:318–21.
4. Demeestere I, Simon P, Emiliani S, Delbaere A, Englert Y. Fertility preservation: successful transplantation of cryopreserved ovarian tissue in young patient treated for Hodgkin's disease. Oncologist 2007;12:1437–42.
5. Andersen C, Loft A, Ernst E, Nyboe A, Rosendahl M. Assisted reproductive techniques after autotransplantation of frozen/thawed ovarian tissue. Hum Reprod 2007;22(suppl 1):i41:O104.
6. Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at −196 C. Endocrinology 1999;140:462–71.
7. Nisolle M, Casanas-Roux F, Qu J, Motta P, Donnez J. Histologic and ultrastructural evaluation of fresh and frozen-thawed human ovarian xenografts in nude mice. Fertil Steril 2000;74:122–9.
8. Aubard Y, Piver P, Cognié Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen–thawed ovarian cortex in sheep. Hum Reprod 1999;14:2149–54.
9. Schubert B, Canis M, Darcha C, Artonne C, Smitz J, Grizard G. Follicular growth and estradiol follow-up after subcutaneous xenografting of fresh and cryopreserved human ovarian tissue. Fertil Steril. In press.
10. Nugent D, Newton H, Gallivan L, Gosden RG. Protective effect of vitamin E on ischaemia-reperfusion injury in ovarian grafts. J Reprod Fertil 1998;114:341–6.
11. Israely T, Nevo N, Harmelin A, Neeman M, Tsafriri A. Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue. Hum Reprod 2006;21:1366–79.
12. Kim SS, Yang HW, Kang HG, Lee HH, Lee HC, Ko DS. Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. Fertil Steril 2004;82:679–85.

13. Gallez B, Baudelet C, Jordan BF. Assessment of tumor oxygenation by electron paramagnetic resonance: principles and applications. NMR Biomed 2004;17:240–62.
14. Khan N, Williams BB, Hou H, Li H, Swartz HM. Repetitive tissue measurements by electron paramagnetic resonance oximetry: current status and future potential for experimental and clinical studies. Antioxid Redox Signal 2007;9:1–13.
15. Gallez B, Mader K. Accurate and sensitive measurements of pO2 in vivo using low frequency EPR spectroscopy: how to confer biocompatibility to the oxygen sensors. Free Radic Biol Med 2000;29:1078–84.
16. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomised sheep by autografts stored at −196°C. Hum Reprod 1994;9:597–603.
17. Dinguizli M, Jeumont S, Beghein N, He J, Walczak T, Lesniewski PN, et al. Development and evaluation of biocompatible films of polytetrafluoroethylene polymers holding lithium phthalocyanine crystals for their use in EPR oximetry. Biosens Bioelectron 2006;21:1015–22.
18. Liu KJ, Gast P, Moussavi M, Norby SW, Vahidi N, Walczak T, et al. Lithium phthalocyanine: a probe for electron paramagnetic resonance oximetry in viable biological systems. Proc Nat Acad Sci U S A 1993;90:5438–42.
19. Dunn JF, Swartz H. In vivo electron paramagnetic resonance oximetry with particulate materials. Methods 2003;30:159–66.
20. Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. Hum Reprod 1986;1:81–7.
21. Baird DT, Campbell B, de Souza C, Telfer E. Long-term ovarian function in sheep after ovariectomy and autotransplantation of cryopreserved cortical strips. Eur J Obstet Gynecol Reprod Biol 2004;113:S55–9.
22. Dolmans MM, Martinez-Madrid B, Gadisseux E, Guiot Y, Yuan Yuan W, Torre A, et al. Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice. Reproduction 2007;134:253–62.
23. Erjavec V, Pavlica Z, Sentjurc M, Petelin M. In vivo study of liposomes as drug carriers to oral mucosa using EPR oximetry. Int J Pharm 2006;307:1–8.
24. Hou H, Grinberg OY, Taie S, Leichtweis S, Miyake M, Grinberg S, et al. Electron paramagnetic resonance assessment of brain tissue oxygen tension in anesthetized rats. Anesth Analg 2003;96:1467–72.
25. Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, et al. Cellular and molecular aspects of ovarian follicle ageing. Hum Reprod Update 2008;14:131–42.
26. Alam H, Maizels ET, Park Y, Ghaey S, Feiger ZJ, Chandel NS. Follicle stimulating hormone activation of hypoxia-inducible factor-1 by the phosphatidylinositol 3-kinase/AKT/Ras homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway is necessary for induction of select protein markers of follicular differentiation. J Biol Chem 2004;279:19431–40.
27. Monniaux D, Pisselet C. Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro. Biol Reprod 1992;46:109–19.
28. Yang H, Heun Lee H, Chang Lee H, Sung Ko D, Kim SS. Assessment of vascular endothelial growth factor expression and apoptosis in the ovarian graft: can exogenous gonadotropin promote angiogenesis after transplantation? Fertil Steril. Published online 21 February 2008 [Epub ahead of print].
29. Wang GL, Jiang BH, Semenza GL. Effect of altered redox states on expression and DNA-binding activity of hypoxia-inducible factor 1. Bioch Biophys Res Commun 1995;212:550–6.
30. Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, et al. Impact of mouse strain differences in innate hindlimb collateral vasculature. Arterioscler Thromb Vasc Biol 2006;26:520–6.
31. Martinez-Madrid B, Donnez J, Van Eyck AS, Veiga-Lopez A, Dolmans MM, Van Langendonckt A. Chick embryo chorioallantoic membrane (CAM) model: a useful tool to study short-term transplantation of cryopreserved human ovarian tissue. Fertil Steril. Published online 21 February 2008 [Epub ahead of print].
32. Dissen GA, Lara HE, Fahrenbach WH, Costa ME, Ojeda SR. Immature rat ovaries become revascularized rapidly after autotransplantation and show a gonadotropin-dependent increase in angiogenic factor gene expression. Endocrinology 1994;134:1146–54.
33. Israely T, Dafni H, Nevo N, Tsafriri A, Neeman M. Angiogenesis in ectopic xenotransplantation: multiparameter characterization of the neovasculature by dynamic contrast-enhanced MRI. Magn Reson Med 2004;52:741–50.
34. Jiang J, Nakashima T, Liu KJ, Goda F, Shima T, Swartz HM. Measurement of pO2 in liver using EPR oximetry. J Appl Physiol 1996;80:552–8.