The effects of whole ovarian perfusion and cryopreservation on endothelial cell-related gene expression in the ovarian medulla and pedicle

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ABSTRACT: Fertility preservation by whole ovarian cryopreservation requires successful cryopreservation of both the ovary and its vascular supply. Previous work has indicated detrimental effects of both perfusion and cryopreservation on the ovarian vasculature. This study assessed the effects of blood perfusion, alone or in combination with cryopreservation, on functional effects in the follicle population and ovarian function in vivo following short-term autotransplantation of the tissue after vascular reanastomosis and measured acute changes in endothelial cell-related gene expression within the ovarian medulla and pedicle. Following autotransplantation for 7 days, primordial, transitional and primary follicle densities were significantly reduced (P < 0.05) and stromal Ki67 and caspase-3 expression significantly increased (P < 0.05) in cryopreserved but not fresh or perfused whole ovaries. There was evidence of clot formation and fluorescent microsphere (FMS) extravasation in the medulla of all cryopreserved ovaries, indicating vascular damage. Utilizing a customized RT–PCR array or conventional RT–PCR, we found that perfusion alone resulted in down-regulation in the expression of caspase 6 and thrombospondin 1 (THBS1) genes in the medulla. Following additional cryopreservation, endothelial nitric oxide synthase (eNOS), endothelin 1, endothelin receptor A and Bcl-2 expression were significantly (P < 0.05) down-regulated. In the pedicle, both perfusion and cryopreservation caused a down-regulation of eNOS and THBS1, and an up-regulation in Bax expression. Perfusion also caused a down-regulation of TNF and up-regulation of endothelin-2 expression (P < 0.05). In conclusion, this study has identified a number of endothelial cell-related genes expressed in the medulla which are acutely affected by both cryopreservation and perfusion, supporting the hypothesis that both interventions have deleterious effects on endothelial cell function.

Key words: ovary / cryopreservation / gene expression / medulla / pedicle

Introduction

Whole ovarian cryopreservation (WOCP) represents an exciting new technique aimed at improving the efficacy of ovarian tissue cryopreservation and fertility restoration. The techniques of cryopreserving and subsequent autografting of ovarian cortical tissue grafts have been developed over several years and are now offered clinically, with increasingly successful results (Anderson et al., 2008; Bedaiwy et al., 2008). Indeed there are now reports of live births derived from previously cryopreserved grafts; resulting from both spontaneous conceptions and conceptions following IVF (Donnez et al., 2004; Meirow et al., 2005, 2007; Demeestere et al., 2007; Andersen et al., 2008; Ernst et al., 2010). Despite this, however, the depleted follicle reserve contained within these grafts coupled with further follicle losses as a result of cryopreservation and grafting means that they provide only a limited window of fertility restoration (Baird et al., 1999; Meirow et al., 2007). In addition, as age plays a critical role in determining the follicle reserve (Faddy et al., 1992; Wallace and Kelsey 2010), the successful use of cryopreserved cortical autografts, in terms of pregnancy and live births, has been limited mainly to younger women in their 20s and early 30s with a total of 18 live births to date (Andersen et al., 2012; Silber, 2012).

As an alternative, whole ovaries may prove to be a superior option to ovarian cortical grafts. The use of whole ovaries complete with the vascular supply could maximize the follicle population preserved and transferred and would also provide the opportunity to immediately
revascularize the transplant, thus reducing the post-graft ischaemic follicle loss. However, the increased mass, complexity and diversity of tissues being cryopreserved raise a number of technical issues. One of these initial problems was the ability to fully permeate the organ with cryoprotectants. This was subsequently solved by cannulating the ovary via the ovarian artery and perfusing it with the cryoprotective media (Bedaiwy et al., 2003) and utilizing a novel directional freezing methodology, workers in Israel have been able to restore ovarian function in three out of eight young ewes <12 months old following WOCP and autotransplantation in lambs (Revel et al., 2004; Arav et al., 2005).

Despite this success, recent findings from our laboratory utilizing slow freezing of whole ovaries from adult animals suggest that both perfusion and cryopreservation may have some deleterious effects on the ovary; in particular on subsequent blood flow through the ovary which may then result in substantial follicle losses (Onions et al., 2008, 2009). The results of one study, which involved the transplantation of either fresh media perfused or cryopreserved whole ovaries suggested that perfusion alone resulted in significant follicle losses despite apparent long-term vascular patency following transplantation (Onions et al., 2009). The findings from this study also postulate that cryopreservation of the vascular pedicle may cause microvascular damage with the evidence of microvascular hemorrhages in the autotransplanted pedicle. These findings led us to hypothesize that perfusion either alone or in combination with cryopreservation may have a detrimental effect on the ovarian vasculature, and in particular on the ovarian microvasculature within the ovarian medulla, which may then impact on follicle survival.

Targeted, low-density, high-sensitivity real-time PCR micro-arrays are now available commercially to reproducibly quantify the expression profile of multiple genes and pathways which are up- or down-regulated by imposed treatments. On the basis of our previous findings and hypotheses, we utilized a customized RT–PCR array to quantify the expression profile of replicated panels of specific genes associated with endothelial cell biology. In addition, the relative expression levels of endothelial nitric oxide synthase (eNOS), a powerful vasodilator (Cooke and Dzau, 1997), and endothelin 1 (ET-1), which causes vasoconstriction (Agapitov and Haynes, 2002), were measured using conventional real-time PCR (RT–PCR) as these genes were not represented on the array but were considered highly relevant. These arrays and RT–PCR targets were used to measure the impact of the experimental treatments detailed below on vascular tissue tone and function within the ovarian medulla and pedicle. The potential subsequent impact of these alterations to the ovarian macro- and microvascular system on ovarian tissue and follicle survival were assessed by studying follicle population densities and stromal Ki67 expression as an indicator of cellular proliferation and health, and caspase 3 expression as a measure of cellular apoptosis, within both ovarian follicles and stromal cells in tissue following transplantation. In addition, potential microvascular damage within the medulla was evaluated following the perfusion of fluorescent microspheres of varying sizes through the ovary and assessing the size and extent of FMS extravasation from vessels within the medulla.

Materials and Methods

All materials were purchased from Sigma Aldrich, Dorset, UK, unless otherwise stated. All sutures were purchased from Ethicon, Edinburgh, UK. All animal procedures were carried out with approval from the UK Home Office and in accordance with the Animals (Scientific Procedures) Act 1986.

Study 1: Acute effects of perfusion or cryopreservation on autotransplanted ovarian blood supply, microvascular integrity and follicle population

Left ovaries complete with a 10–15 cm length of the associated pedicle were removed from 6-year-old mature multiparous Greyface ewes (n = 12) under anaesthesia during the breeding season. Ovaries were either (i) immediately autotransplanted to the original ovarian vasculature (n = 4; Fresh group); (ii) perfused with vehicle media [Leibovitz L-15 media supplemented with 10% [v/v] fetal bovine serum (FBS)], following ovarian arterial cannulation, for 60 min at a rate of 0.5 ml min⁻¹ before being autotransplanted to the original ovarian vasculature (n = 4; perfused group); or, following ovarian arterial cannulation, were cryoperfused and cryopreserved following the method described previously both in this paper and elsewhere (Onions et al., 2008). Four weeks after cryopreservation, ovaries were thawed, using the method as described previously (Onions et al., 2008) and autotransplanted to the contralateral ovarian artery and vein (n = 4; cryopreserved group). Vascular reanastomoses of the artery and vein were achieved using 8/0 and 10/0 Ethilon sutures, respectively, and with the aid of a Zeiss-operating microscope.

Seven days after autotransplantation, the transplants were examined in situ for signs of ovarian health and, more importantly, blood flow to and from the ovarian transplant. The transplants were then removed from all the ewes. The ovarian arteries were cannulated and the ovaries perfused with a 3 ml suspension of fluorescent microspheres (FMS, 10 μm, 1 μm and 100 nm diameter; Duke Scientific Corps, Palo Alto, CA, USA). Ovaries were then tied off and cut from the pedicle and hemisectioned. One half of the ovary was fixed in 4% paraformaldehyde for subsequent FMS extravasation analysis and from the other, samples of ovarian cortex were taken and fixed in Bouin’s fixative for subsequent analysis of follicle counts and stromal Ki67 and Caspase 3 expression.

Histological Analyses

The fixed tissue samples were processed using an ascending series of alcohols and cleared using either xylene (Bouin’s fixated tissue) or Histo-Clear II (National Diagnostics, GA, USA; FMS fixed tissue). The tissue samples were then paraffin wax embedded for subsequent histological evaluation.

FMS analysis

10-μm sections (n = 2 per ovary) of the hemi-ovaries processed for FMS analysis were mounted on superfrost slides and viewed directly using green (10 μm FMS), red (1 μm FMS) and blue (100 nm FMS) fluorescence microscopy utilizing a Leica DMRB microscope fitted with a Hamamatsu Digital Camera. Blood vessels within the ovarian medulla were studied for evidence of FMS extravasation from the lumen of the vessels (Baffert et al., 2006).

Follicle counts

Ten serial 5-μm sections of ovarian cortical tissue from from three different regions of the ovary, at least 100 μm apart, were stained with haematoxylin and eosin. Within these sections, the numbers of follicles present were recorded and classified as primordial, transitional, primary, secondary, pre-antral or antral, in a similar way to that described previously (Lundy et al., 1999). As the size of cortical tissue section varied between animals/samples, the number of fields of view over which the follicles had been counted was also recorded and the follicle counts expressed as the
Whole ovary cryopreservation and gene expression

Study 2: Blood perfusion effects on endothelial cell-related gene expression

Ovarian perfusion, cryopreservation and tissue sample collection

To examine the possible deleterious effects of ovarian perfusion and/or cryopreservation on the ovarian vasculature in more detail, the effect of these interventions on endothelial cell-related gene expression was determined. Both left and right ovaries complete with a 10–15-cm length of ovarian pedicle were recovered from 6-year-old mature Greyface parous ewes (n = 4) immediately after sacrifice during the breeding season. Jugular blood was also collected from each ewe into labelled heparinized pots (100 IU ml\(^{-1}\) heparin sodium, Wockhardt UK Ltd, Wrexham, UK; 2 pots per ewe) for subsequent ovarian perfusion in order to mimic the in vivo exposure and reaction to re-perfusion observed in Study 1.

The ovarian arteries were cannulated using 2.5 F (0.75 mm OD) intravenous cannulae (SimS Portex Limited, Kent, UK) and tied securely in place using 0 mersilk (Ethicon, Edinburgh, UK). Right ovaries were immediately perfused, via the cannula, with 10-ml heparinized blood taken from its donor ewe at a rate of \(\sim 1\) ml min\(^{-1}\) either manually or using a syringe-driven perfusion pump fitted with a 20-ml syringe (Precidor Infors Ag Basel; ChemLab Scientific Products Ltd, Hornchurch, UK). Following blood perfusion, the ovary and vasculature were flushed with Ringer’s solution at a rate of 1 ml min\(^{-1}\) (∼5 ml; 142 mM NaCl, 4 mM KCl, 2 mM NaHCO\(_3\), 50 μM MgCl\(_2\), 2 mM CaCl\(_2\)) before samples were taken from the ovarian pedicle and medulla and snap frozen in liquid nitrogen for subsequent gene expression analysis.

The left ovaries and cannulated pedicle sections from each ewe were transferred to 15-ml cryogenic vials (Scientific Laboratory Supplies, Nottingham, UK) and cryopreserved following a slow-freezing protocol, using a controlled rate Planer freezer (Kryo 550-16, Planer Products, Nottingham, UK) and cryopreserved following a slow freezing protocol, using a controlled rate Planer freezer (Kryo 550-16, Planer Products Ltd, Sunbury-on-Thames, UK) before being transferred to liquid nitrogen storage. The following day, the ovaries were thawed using a rapid thawing protocol, again as described previously (Onions et al., 2008). Each ovary was then perfused with 10-ml heparinized blood, which had been previously taken from its donor ewe and refrigerated overnight. The ovary and vasculature were then flushed clear with heparinized Ringers (∼5–15 ml) before samples were taken from the ovarian pedicle and medulla and snap frozen in liquid nitrogen for subsequent gene expression analysis.

In addition, the right ovary complete with the vascular pedicle was also removed from a further four parous ewes. Samples of the ovarian pedicle and medulla were taken immediately after removal and snap frozen. The tissue was collected and analysed as time 0 control tissue.

Tissue preparation and mRNA extraction

Frozen tissue samples were prepared for mRNA extraction by grinding to a powder in liquid nitrogen using a pestle and mortar and lysing in RLT buffer (QIAGEN Ltd, West Sussex, UK) containing 1% v/v β-mercaptoethanol. Tissue lysates were then homogenized using a QIAshredder (QIAGEN Ltd, West Sussex, UK) and mRNA was extracted from the homogenized tissue lysates using the RNaseasy mini kit (QIAGEN Ltd, West Sussex, UK) with the one-column DNase digestion step carried out.

Custom RT–PCR Arrays

Single-strand cDNA was synthesized from 1-μg high-quality mRNA using the First Strand cDNA synthesis kit (SA Biosciences, Maryland, USA). The custom PCR array was prepared and run according to the kit instructions (SA Biosciences, MD, USA). Details of the genes identified by the custom plates are given in Table 1. Hypoxanthine phosphoribosyltransferase 1 was included on the PCR plate as a housekeeping gene in addition to reverse transcription control (RTC) and positive control (PPC) wells. Gene expression from three samples was amplified per PCR plate using the following amplification programme: 10 min at 95°C to activate the

Follicle count data were analysed using a generalized linear regression on the GenStat statistical computer package (VSN International Ltd, Hemel Hempstead, UK) using a Poisson distribution following a logarithmic transformation.

Ki67 immunohistochemistry

Serial 5-μm sections of ovarian cortical tissue 100 μm apart (n = 2) were stained for either Ki67 expression utilizing the Bond-Max™ (Leica Microsystems (UK) Ltd) automatic immunohistochemical stainer using an anti-Ki67 mouse monoclonal antibody (85 μg ml\(^{-1}\); Vector Laboratories, Peterborough, UK), diluted 1:100 in antibody diluent (Leica Microsystems (UK) Ltd).

Following staining, random images were taken of the ovarian stroma for each tissue section (n = 3 per tissue section) using ‘Volocity’ image analysis software (Improvision®, PerkinElmer; Cambridge UK). All positively stained and counterstained cells in each image were counted, again using the ‘Volocity’ image analysis software, and the percentage of cells expressing Ki67 was then calculated. Data were found to be not normally distributed and the comparison between time 0 and post-treatment tissue was performed utilizing the Wilcoxon-signed rank test.

Caspase 3

Serial 5-μm sections were cut from ovarian tissue blocks and these were mounted onto SuperFrost® Plus microscope slides (Menzel-Glaser, Braunschweig, Germany) and baked overnight at 45°C. The sections were dewaxed and rehydrated before being placed in 0.01 M citrate buffer and heated in a microwave for epitope retrieval. Non-specific staining was blocked using normal donkey serum (Sigma D9663; Dorset UK) before the addition of Caspase 3 primary antibody (Abcam ab405; Cambridge UK), which recognizes the cleaved form of the molecule as a marker for apoptosis. The primary antibody was then followed by FITC-labelled secondary antibody (Donkey anti rabbit IgG FITC Santa Cruz sc2090; California USA) and after a wash step the sections were counterstained with DAPI (Leica Microsystems (UK) Ltd).

The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of primary antibody on tissue from the same animal. Positive controls consisted of sections of ovarian tissue from another animal which were included in each run. The slides were observed utilizing a Nikon Eclipse 90i microscope and images of stroma in identical areas of the sections were taken using Volocity Acquisition Software, at wavelengths of 518 nm for FITC staining and 460 nm for DAPI counterstaining. Analysis of the images was carried out using the Volocity Quantification Software in which the number of Caspase 3 positive stained granulosa and theca cells was counted and the total number of cells within the image counted on the DAPI counterstained image. Data were found not to be normally distributed and the comparison between time 0 and post-treatment tissue was performed utilizing the Wilcoxon-signed rank test.

Number of follicles per field of view (Onions et al., 2009). Primordial and transitional follicle counts were combined for statistical analysis.
HotStart DNA polymerase followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analysed using the data analysis template supplied by SA Biosciences and which utilized Student’s t-test for determining statistical significance.

Reverse transcription and conventional real-time PCR
Single-strand cDNA was synthesized from 1 μg mRNA using random primers (Promega, Southampton, UK) and the reverseTIT cDNA synthesis kit (Invitrogen, Paisley, UK). The RT programme consisted of 70°C for 5 min, 47°C for 35 min and inactivation at 75°C for 10 min.

Table I List of genes in the customized endothelial cell biology array

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Official full name</th>
<th>Ref Seq #</th>
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<tr>
<td>AGT</td>
<td>Angiotensinogen</td>
<td>NM_000029</td>
</tr>
<tr>
<td>ANGPT1</td>
<td>Angiopoietin 1</td>
<td>NM_001146</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>NM_004324</td>
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<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>NM_000633</td>
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<tr>
<td>BCL2L1</td>
<td>BCL2-like 1</td>
<td>NM_138578</td>
</tr>
<tr>
<td>CASP6</td>
<td>Caspase 6</td>
<td>NM_032992</td>
</tr>
<tr>
<td>CFLAR</td>
<td>CASP8 and FADD-like apoptosis regulator</td>
<td>NM_003879</td>
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<tr>
<td>CSF2</td>
<td>Colony-stimulating factor 2</td>
<td>NM_000758</td>
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<td>EDN2</td>
<td>Endothelin 2</td>
<td>NM_001956</td>
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<td>Endothelin receptor type A</td>
<td>NM_001957</td>
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<tr>
<td>FASLG</td>
<td>Fas ligand</td>
<td>NM_000639</td>
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<td>ITL</td>
<td>Interleukin 7</td>
<td>NM_000880</td>
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<tr>
<td>ITGA5</td>
<td>Integrin, alpha 5</td>
<td>NM_002205</td>
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<td>Integrin, beta 1</td>
<td>NM_002211</td>
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<td>ITGB3</td>
<td>Integrin, beta 3</td>
<td>NM_000212</td>
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<td>NPR1</td>
<td>Natriuretic peptide receptor A/ guanylate cyclase A</td>
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<td>PECAM1</td>
<td>Platelet/endothelial cell adhesion molecule</td>
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<td>PF4</td>
<td>Platelet factor 4</td>
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<td>Phospholipase A2, group IVC</td>
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</tr>
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<td>PTGIS</td>
<td>Prostaglandin I2 (prostacyclin) synthase</td>
<td>NM_000961</td>
</tr>
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<tr>
<td>SELPLG</td>
<td>Selectin P ligand</td>
<td>NM_003006</td>
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<td>SOD1</td>
<td>Superoxide dismutase 1, soluble</td>
<td>NM_000454</td>
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<tr>
<td>TEK</td>
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</tr>
<tr>
<td>THBS1</td>
<td>Thrombospondin 1</td>
<td>NM_003246</td>
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<tr>
<td>TIMP1</td>
<td>TIMP metalloproteinase inhibitor 1</td>
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<td>Tumor necrosis factor receptor superfamily, member 10c</td>
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<td>RTC</td>
<td>Reverse transcription control</td>
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</tr>
<tr>
<td>HGDC</td>
<td>Human genomic DNA contamination</td>
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eNOS and ET-1 expression was semi-quantified relative to 18S expression by real-time PCR using the 7500 Fast PCR system (Applied Biosystems, Inc., CA, USA). Oligonucleotide primers for eNOS, ET-1 and 18S RNA were synthesized in house using sequences published previously; eNOS (Farrow et al., 2008) sense: 5′-CCTCAGCGCTACACATTCC-3′; antisense: 5′-GCAAAGCGAGTCTGTA-3′; ET-1 (Klipper et al., 2004) sense: 5′-TGTTCTTCATGAGCCT-3′; antisense: 5′-TCAGCTTGCAACACGAA-3′; 18S sense: 5′-TTGACTCAACGCGGAAATCT-3′; antisense: 5′-AGAAAGAGATCATTCAATGCTACT-3′.

The PCR conditions used were the same for all primers sets; the amplification programme consisted of an initial activation step at 95°C for 15 min followed by 40 cycles of 20s denaturation at 95°C, 30 s annealing at 60°C and 1 min extension at 72°C. Relative expression levels were measured using a standard curve constructed using cDNA synthesized from ovine endothelial cells isolated from the aortic arch as both eNOS and ET-1 are highly expressed in endothelial cells (Inoue et al., 1989; Shaul, 2002). Standards and samples were amplified in duplicate and eNOS and ET-1 expression levels were normalized against differences in starting amount using the 18S endogenous control. Data were analysed using a two-tailed Student’s t-test assuming equal variances to determine statistical significance.

Results

Study 1: Acute effects of perfusion or cryopreservation on autotransplanted ovarian blood supply, microvascular integrity and follicle population

Post-transplant observations
Blood supply to both the fresh and perfused ovarian transplants appeared unaffected with good flow, both immediately following arterial reanastomosis and on recovery 7 days post-transplant. Ovaries which had been cryopreserved prior to transplantation, however, whilst having good immediate patency, on recovery of the transplant after 1 week, the ovarian artery was black with clotted blood and blood flow had arrested in 3/4 ewes. In the fourth ewe, the pedicle had a ‘normal’ appearance until removal of the transplant was started, thereafter the artery rapidly turned black, indicating blood clotting. Subsequent perfusion of FMS was unsuccessful in all cases and on semi-section of the ovary, the medulla contained blood clots.

FMS perfusion
Observations of the patterns and locations of the FMS following perfusion of the 10 μm, 1 μm and 100 nm FMS through the ovary 7 days after transplantation were carried out within the ovarian medulla. The larger two sizes of FMS (10 μm and 1 μm) did not show any evidence of extravasation and were always seen within the lumen of the blood vessels. The large, 10 μm FMS tended to line the walls of the blood vessels, without leaking through the endothelium. In contrast, the smallest FMS used (100 nm) did show signs of leakage through the vessel wall. In all the fresh ovarian medulla studied, there was only one distinct band of blue fluorescence observed (indicating the presence of the 100 nm FMS), lining the endothelial wall of the blood vessels (Fig. 1A). However, in ∼50% of the perfused ovarian medullae and 80–90% of cryopreserved ovarian medullae, two distinct bands of blue colour were observed.
Indicating that some of the 100 nm FMS were able to cross the endothelial layer ‘barrier’ and penetrate further into the smooth muscular wall of the blood vessel.

Despite relatively large reductions in primordial and transitional follicle densities, these differences were not found to be significantly different following autotransplantation in either fresh control or perfused ovary treatment groups (Fig. 2). However in cryopreserved ovaries, there was a significant loss of primordial and transitional follicles 7 days following autotransplantation when compared with time 0 from the same animals, primordial/transitional follicle density fell by 96% from 0.42 to 0.02 ($P < 0.001$). Consequently, primary follicle densities in cryopreserved ovaries following autotransplantation were also found to be significantly lower than those found in autotransplanted fresh and perfused ovaries ($P < 0.001$; Fig. 2).

In terms of the primary follicle population, both fresh non-perfused and cryopreserved ovaries sustained large declines in numbers (63% loss and 95% loss, respectively), however only the loss of follicles in the cryopreserved ovary group following autotransplantation was found to be significantly reduced ($P < 0.005$). Conversely, ovaries which had been perfused with media prior to being autotransplanted showed a non-significant increase in the density of primary follicles 7 days after transplantation from 0.035 FPFV to 0.039 FPVF; a 10% rise.

Count data from the larger follicles, secondary, pre-antral and antral, were not included in the statistical analysis due to very low numbers seen; however, these larger follicles were invariably absent from post-transplant cortical tissue.

Positive Ki67 expression within the general ovarian stromal cell population was similar in time 0 cortical tissue between treatment groups and following autotransplantation of ovaries for 7 days, the percentage of stromal cells staining positive for Ki67 showed a trend for higher levels of expression with increased degree of intervention (Figs 3A and 4A–C). Thus, the proportion of stromal cells expressing Ki67 before and after intervention displayed an increase that approached statistical significance for fresh and perfused tissue ($P < 0.07$), whereas tissue that had been perfused and cryopreserved had significantly more Ki67-positive stromal cells ($P < 0.05$) than at time 0.

Caspase 3 expression

Caspase 3 expression within the general ovarian stromal cell population was similar in time 0 cortical tissue for non-perfused, fresh perfused and cryopreserved groups (Figs 3B and 4D–F) but differed...
markedly in their response to experimental intervention. Thus, in fresh non-perfused and fresh perfused tissue the proportion of caspase 3-positive cells tended to decline relative to untreated tissue but exhibited a significant increase (P < 0.05) in tissue that had been perfused and cryopreserved prior to autotransplantation (Fig. 3B).

**Study 2: Blood perfusion effects on endothelial cell-related gene expression**

**Perfusion observations**

In contrast to both the cryopreservation media and Ringer’s solution, during perfusion of blood through the ovary and pedicle, resistance to perfusion gradually increased. This was particularly true for the left ovaries which had previously been cryopreserved, and in these ovaries flow eventually became blocked and could not be cleared by subsequent flushing with Ringer’s solution. When these ovaries were bisected during sample retrieval, the ovarian medulla was red/black with blood. Whilst the right ovaries, perfused fresh with blood, were felt to offer increased resistance to blood perfusion, flow was never occluded and the ovarian vasculature was able to be flushed clear with Ringer’s solution.

**Real-time PCR**

Perfusion alone had no significant effect on eNOS expression within the medulla (Fig. 5A); however, it did cause a significant down-regulation of eNOS expression in the pedicle (P < 0.05; Fig. 5B). Perfusion of blood alone appeared to suppress ET-1 mRNA expression in the ovarian medulla (Fig. 5A); however, these effects were not found to be statistically significant, nor was there any significant effect seen in the pedicle (Fig. 5B).

Cryopreservation of ovaries followed by blood perfusion resulted in the significant down-regulation of both eNOS and ET-1 mRNA expression within the ovarian medulla (P < 0.05; Fig. 5A); however in the pedicle, only eNOS expression was significantly attenuated by freeze-thawing (P < 0.05; Fig. 5B).

**Custom RT–PCR array**

In the ovarian medulla, perfusion alone resulted in a significant down-regulation of the expression of caspase 6 (CASP6) and thrombospondin 1 (THBS1) mRNA expression (P < 0.05). Following cryopreservation, endothelin receptor A (EDNRA) and Bcl-2 expression were significantly (P < 0.05) down-regulated (Fig. 6).

In the pedicle, both perfusion alone and in combination with cryopreservation caused a significant up-regulation of Bcl-2-like X protein (Bax) expression and down-regulation of THBS1 (P < 0.05). Following perfusion alone, endothelin-2 (EDN2) was significantly up-regulated, whilst tumour necrosis factor (TNF) expression was significantly down-regulated (P < 0.05; Fig. 6).

**Discussion**

The results from this study provide compelling evidence to support the hypothesis that both perfusion per se and perfusion and cryopreservation of the ovary and its vascular pedicle result in deleterious changes to the endothelial cells of the ovarian vasculature that could result in ischaemic damage to both the ovarian stroma and the population of primordial and growing follicle. Further, it was evident from the results that the effects of perfusion and cryopreservation on gene expression differed between the medulla and the pedicle. This implies that even within ‘vascular tissue’, the different tissue types of macrovasculature (of the pedicle) and microvasculature (of the medulla) have different responses to perfusion and cryopreservation. Consequently, these tissue types are likely to have different requirements and optimal conditions for cryoperfusion, cryopreservation and thawing, emphasizing the complexity of producing a cryopreservation protocol suitable for the diverse range of tissue types within the whole ovary and associated vascular pedicle.

Gene expression associated with vascular tone regulatory pathways was affected by both perfusion and cryopreservation. Expression of protein for eNOS (Grazul-Bilska et al., 2006), ET-1 (Girsh et al., 1996; Bridges et al., 2011), THBS1 (Greenaway et al., 2005), EDNRA (Bridges et al., 2011), EDN2 (Bridges et al., 2011), TNF (Murdoch et al., 1997), Bcl-2 and Bax (Liu et al., 2009; Matsuda et al., 2012) has been previously reported to be associated with the ovarian vasculature in a number of monovulatory species. Although a number of
these factors, including eNOS (Grazul-Bilska et al., 2006), the endothe-
lins and their receptors (Bridges et al., 2011) and TNF (Murdoch et al., 1997), are also expressed by follicular somatic cells present in the ovarian cortex, the restriction of the gene analysis to the vascular ovarian medulla and pedicle suggests that the main effects observed are restricted to vascular cell types. eNOS expression levels in both the medulla and the pedicle were affected, but in response to seemingly different triggers. In the medulla, eNOS expression was significantly down-regulated by cryopreservation but not perfusion, whereas in the pedicle the down-regulatory effect appeared to be mediated by blood perfusion with no further down-regulation caused by additional freeze-thawing. This may suggest that the many small-branching vessels in the medulla are able to disperse or accommodate any changes in the pressure created by perfusion to limit the effects on eNOS expression. However, these small blood vessels may be more sensitive to effects caused by cryopreservation. In contrast, the larger ovarian artery in the pedicle is the primary vessel directly exposed to the potential effects of perfusion. Whilst the blood pressure within the ovarian artery is, to the authors’ knowledge, not known, blood flow is estimated to be $\approx 1.7 \text{ ml min}^{-1}$ (Rabiee et al., 1997). This is higher than the perfusion rate used in this study which was 1 ml min$^{-1}$. Therefore, it may be assumed that blood pressure was also reduced below normal levels. A reduction in eNOS levels leads to hypertension by preventing vasodilation (Shaul, 2002) and therefore the diminished eNOS expression seen in this study may have been triggered by the hypotension created from the artificial perfusion to increase intraluminal pressure as part of a homeostatic mechanism.

**Figure 4** Images showing Ki67 (A–C) and Caspase 3 (D–F) protein expression in ovarian cortical stromal tissue collected 7 days following fresh whole ovarian autotransplantation (A, D), fresh perfusion and autotransplantation (B, E) and WOCP, thawing and autotransplantation (C, F). Cells stained positive for Ki67 are brown; compared with the blue counterstained cells. Caspase-positive cells fluoresce bright green. Magnification x200, scale bars indicate 70 $\mu$m.
ET-1 is one of the most potent vasoconstrictors (Masaki et al., 1991) and expression/production is increased, amongst other things, by the actions of various vasoactive hormones, hypoxia and increased shear stress (Agapitov and Haynes, 2002). Despite the trend of increased ET-1 expression following perfusion, the lack of significant effect does suggest that the change in perfusion pressure generated was insufficient to trigger significant vasoconstriction by this mechanism. Similar to eNOS, ET-1 expression was unaffected by cryopreservation in the pedicle; however, in the medulla it was significantly down-regulated. This again suggests that the smaller microvascular vessels within the medulla are sensitive to the effects of cryopreservation, reducing their responses over vascular tone.

In contrast to ET-1, ET-2 expression was significantly up-regulated in the pedicle following perfusion. Endothelins, including ET-2, are significantly up-regulated as a result of hypoxia (Kourembanas et al., 1991; Elton et al., 1992; Grimshaw et al., 2002; Klipper et al., 2010) and this results therefore suggests that hypoxic conditions may have been created in the ovarian pedicles during perfusion; however, this idea is not supported by the lack of significant effects on ET-1 expression. Endothelin receptor A, which has the binding affinity for both ET-1 and ET-2, was significantly down-regulated in response to cryopreservation in the medulla, with no similar effect in the pedicle. EDNRA is primarily located on vascular smooth muscle cells and binding with either ET-1 or ET-2 causes vasoconstriction of these cells (Agapitov and Haynes, 2002). Reduced expression would therefore limit the vasoconstrictive response of these cells. Expression of EDNRA is, in part, regulated by endothelin itself (Rapoport and Zuccarello, 2011) and therefore its down-regulation could be as a result of the effects of cryopreservation on ET-1 expression (Fig. 5). This theory is supported by the effects, or

**Figure 5** Relative expression of eNOS and ET-1 in ovarian medulla (A) and pedicle (B) tissue at time 0 (black bars), following fresh ovarian perfusion with autologous blood (grey bars) and following ovarian cryopreservation and subsequent perfusion with autologous blood (white bars). Significant differences in the expression between treatments are denoted by *(P < 0.05).

**Figure 6** Graph to show the fold change in relative gene expression of the genes whose expression was significantly *(P < 0.05)* affected by either blood perfusion alone (black bars) or WOCP followed by blood perfusion (grey bars).
lack thereof, on ET-1 expression and therefore also on EDNRA expression levels in the pedicle in response to cryopreservation.

The gene expression changes seen in response to cryopreservation and perfusion tend to show a promotion of cellular apoptosis (Fig. 6), with an up-regulation of pro-apoptotic Bax expression and down-regulation of anti-apoptotic Bcl-2 expression, suggesting detrimental effects of both of these processes on cell survival. Both Bax and Bcl-2 act at the level of the mitochondria to determine the release of cytochrome c, one of the earliest events in triggering the apoptotic cascade (Zimmermann and Green, 2001). In contrast, however, in the medulla, Casp6 expression was down-regulated in response to perfusion. CASP6 is an effector caspase and is one of the most downstream caspases of the cascade. This suggests, therefore, that following perfusion within the medulla in particular, despite potential activation of apoptosis by Bax, the final execution of cell death may be suppressed. The reason for this is unclear.

THBS1 expression was down-regulated in response to perfusion in both the medulla and pedicle, and was the only gene response to be common to both tissue types. Whilst expression was also down-regulated in the pedicle in response to cryopreservation, the response was not significantly different from perfusion alone, implying the effect was mediated primarily by perfusion. The down-regulation of THBS1 is most commonly associated with conditions of angiogenic stimulation and wound repair and therefore this effect may imply that perfusion has created an environment where wound repair is necessary. This, coupled with the down-regulation of Casp6 and increase in stromal Ki67 expression, would suggest that perfusion may trigger early wound repair and tissue regeneration pathways. Alternatively, in glialblastoma cells THBS1 has been shown to be down-regulated by anoxia and therefore may also indicate that, as was also potentially shown by the up-regulation of ET-2 expression, that perfusion leads to low or an absence of oxygen (Tenan et al., 2000). In terms of the potential effects of this down-regulation of THBS1 expression, THBS1 is also involved in regulating vascular tone. It has been shown to antagonize the effects of nitric oxide to prevent or limit the vasodilatation of contracted vascular smooth muscle cells (Isenberg et al., 2008). Therefore, this down-regulation of THBS1 may lead to increased vasodilation and blood flow.

Following autotransplantation, previous cryopreservation of the ovary caused a significant decline in the density of primordial, transitional and primary follicles, which was not seen in either of the other two treatment groups. This suggests that WOCP affects follicular cell survival such that widespread follicular apoptosis occurs in the immediate post-thaw/post-transplant period. Whilst this is in agreement with other studies using both cortical patch autographs (Baird et al., 1999; Salle et al., 1999; Campbell et al., 2004) and whole ovarian autotransplants (Imhof et al., 2006; Onions et al., 2009), the cortical graft studies in particular attributed the loss of follicles to the grafting procedure rather than cryopreservation. However the fresh and perfused autotransplants in this study, despite suffering follicle losses, did not sustain significant reductions in follicle density suggesting a freeze-thawing mediated effect on follicle loss in the whole ovary.

In terms of the potential role that cryopreservation-mediated changes in endothelial cell-related gene expression may have played on the loss of the small follicle population; cryopreservation led to the down-regulation of eNOS, ET-1, EDNRA and Bcl-2 expression in the medulla and the down-regulation of THBS1 and up-regulation of Bax expression in the pedicle. The changes most likely to impact follicle survival are those associated with blood flow. However both eNOS and ET-1 and its receptor were down-regulated. Often, if ET-1 levels rise, eNOS levels will increase in line to limit vasoconstriction; therefore if the reverse is also true, these actions would be to limit vasodilatation. The observed effects of the blood perfusion, particularly after cryopreservation, were to increase the resistance to perfusion offered by the ovarian vasculature. In the previously frozen ovaries this went so far as to prevent any further perfusion either of blood, or of the flushing media. If this is also the case in vivo following vascular reanastomosis, it would suggest a blood mediated inhibition of blood flow, potentially from vasoconstriction, which would agree with some of the gene expression changes seen in the medulla. Alternatively, damage to the endothelial cells may initiate blood clot formation and although heparin was included in both in vitro and in vivo studies, more specific anti-thrombotic agents may be required to prevent platelet activation and formation of clots within the ovarian vasculature. Whatever the cause, however, if increased resistance to blood flow does indeed occur in the immediate post-transplant period, this would significantly limit blood flow to the ovary and could cause the acute, large-scale loss of follicles observed in the cryopreserved group in this and other studies (Onions et al., 2009).

In addition to loss of follicles, changes in both Ki67 and caspase 3 protein expression were observed in the ovarian stroma of cryopreserved and autotransplanted ovaries. Ki67 is a marker of cell proliferation and indication of health (Gerdes et al., 1984). Surprisingly, WOCP was seen to significantly increase stromal cell proliferation in the cortical tissue of autotransplanted ovaries. The presence of proliferating, and therefore potentially healthy, stromal cells would indicate healthy stromal tissue which had successfully survived freeze-thawing and would provide good structure and support for developing follicles. However, the significant increase in proliferation over that seen in the fresh control transplanted ovaries, showing deviation from the normal state, may not necessarily be viewed as positive. Increased proliferation may indicate some injury to the stroma had occurred where tissue regeneration is needed; however it may also indicate some genomic damage to the cell or perturbation to the control of the cell cycle. One of the explanations for several of the gene expression changes reported was potential hypoxia. Ki67 expression, as a marker of cell proliferation, has been shown to be increased following both short- and longer term exposure to hypoxia (Paddenberg et al., 2007; Xu et al., 2009) and therefore these results may also support the theory that hypoxic conditions are generated in these whole ovarian autotransplants. This interpretation is supported by a significant increase in caspase 3 expression, a factor that acts towards the end of the apoptotic cascade, in tissue that had been frozen and autotransplanted.

In conclusion, the studies presented in this paper confirm that WOCP and autotransplantation results in deleterious changes to both the ovarian follicle population and surrounding stromal tissue that can be attributed, at least in part, to acute loss of vascular patency (Study 1) that appears to be associated with significant changes in endothelial cell-related gene expression associated with vascular tone, wound repair and/or hypoxia that were induced by both ovarian perfusion alone and the combination of perfusion and cryopreservation (Study 2). This study has therefore identified key
markers which will now allow more focused approaches to improve the success of WOCP by reducing the short-term vascular patency issues in order to improve and maintain blood flow to the transplanted ovaries in the immediate post-surgical period. By achieving this it is hoped that we will reduce follicle loss and improve long-term ovarian function, making WOCP a viable clinical option for patients at risk of premature primary ovarian insufficiency.

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Authors’ roles

V.O. (post-doctoral scientist) performed majority of procedures and prepared first draft MS; R.W. (grant holder) contributed to study conception and MS preparation; C.P.A. (research assistant) contributed to assisted analytical procedures; H.M.P. (grant holder study) contributed to conception and MS preparation; B.K.C. (PI) performed surgical procedures, and contributed to study conception and MS preparation.

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Conflict of interest

None declared.

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