Ovarian endocrine profile and long-term vascular patency following heterotopic autotransplantation of cryopreserved whole ovine ovaries

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BACKGROUND: This study examined the ability of cryopreserved whole ovine ovaries to resume function in vivo following autotransplantation.

METHODS: Swaledale ewes had their left ovaries removed and either perfused but not cryopreserved (n = 4; control), or perfused and cryopreserved (n = 8; cryopreserved) before autotransplantation sub-cutaneously to the neck by microvascular anastomosis. Right ovaries were removed and fixed as non-grafted controls. Weekly jugular venous blood samples were analysed for plasma FSH, LH, inhibin A and progesterone levels, grafts were scanned transdermally and oestrus was detected. Vascular patency was assessed post-mortem and follicle populations were measured in recovered tissue.

RESULTS: Immediate vascular patency was achieved in all ewes and maintained in 7/8 cryopreserved and 3/4 control grafts. Functional corpora lutea were identified in three ewes (one control; two cryopreserved) 18–25 weeks after grafting. Inhibin A levels indicated resumption of follicular development in four cryopreserved and one control ewes, however, castrate gonadotrophin levels persisted in five cryopreserved and two control ewes. Primordial follicle density was reduced following grafting in both cryopreserved and non-frozen ovaries (P < 0.001).

CONCLUSIONS: In conclusion, these results demonstrate successful partial restoration of ovarian function following cryopreservation of the whole ovary and vascular pedicle in a large monovulatory species. The inability to restore full ovarian function was related to loss of primordial follicles rather than vascular patency in both frozen and fresh tissue, suggesting that factors associated with cannulation and perfusion may contribute to this depletion. Further work is therefore needed to elucidate these factors before the procedure could be considered a viable option for fertility preservation.

Key words: cryopreservation / whole ovary / follicle survival / vascular patency / ovarian graft

Introduction

Whole ovarian cryopreservation (WOCP) and autotransplantation represents a potentially valuable technique in the preservation and restoration of fertility in women at risk of primary ovarian insufficiency (POI); a condition first described by Albright et al. (1942) (Welt, 2008). This loss of ovarian function, and consequent infertility and sex hormone deficiency, can have far-reaching clinical and psychological effects such as failure to develop secondary sexual characteristics, loss of self confidence, depression and increased risks of osteoporosis and heart disease (Apperley and Reddy, 1995; Kim et al., 2001; Carr, 2003; Luborsky et al., 2003; Nelson, 2009), and therefore efforts to prevent POI are of high importance.

The first successes in the preservation of follicles following cryopreservation and thawing of ovarian tissue were reported in rodents in the 1950s (Parkes and Smith, 1953; Deansley, 1954; Green et al., 1956) and this was followed by a live birth from mice given previously cryopreserved isografts (Parrott, 1960). However, it was a further 40 years before the first live birth was reported in a large animal species following the grafting of previously cryopreserved cortical strips of ovarian tissue in sheep (Gosden et al., 1994).

Despite this apparent success, the cortical strips grafted in these, and other, studies have been shown to have a limited functional lifespan (Baird et al., 1999; Salle et al., 2003). This is not unexpected as cortical patches contain a reduced number of primordial follicles and around 70% of these follicles are then lost as a result of cryoinjury.
and ischaemia during graft revascularization (Baird et al., 1999). In contrast, WOCP with vascular reanastomosis represents a potentially superior alternative as this technique not only allows the preservation and transfer of the entire follicle reserve, but also allows complete and immediate revascularization and thus the potential for complete restoration of ovarian function.

WOCP and autotransplantation in small animal species such as rats and mice has been carried out relatively successfully for many years (Gunasena et al., 1997; Candy et al., 2000; Wang et al., 2002; Yin et al., 2003). However, in larger species such as sheep and humans, the increased size, mass and metabolic demands of the ovary make the technique more challenging and have necessitated the use of cryoprotectant perfusion through the ovary and microvascular reanastomosis during transplantation. Thus, in contrast to autografts, WOCP has the added technical requirement of ensuring both primordial follicle survival and vascular patency.

Research in both sheep and humans has found that ovarian tissue viability is not significantly affected following WOCP (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2004; Arav et al., 2005; Onions et al., 2008) suggesting that oocytes should be able to resume folliculogenesis and steroidogenesis following autotransplantation. Despite these encouraging in-vitro findings and some live births following WOCP in sheep (Imhof et al., 2006) following autotransplantation, many cryopreserved whole ovarian grafts fail within a relatively short period of time (Bedaiwy et al., 2003; Arav et al., 2005). This has been associated in some studies with vascular tissue damage following cryopreservation of the ovarian pedicle (Courbiere et al., 2005; Onions et al., 2008). However, as none of these large animal model in-vivo studies included groups in which ovaries were perfused and grafted fresh, it is not possible to distinguish between the effects of perfusion, cryopreservation and surgery in contributing to the high failure rate.

In sheep, we have used heterotopic autotransplantation of the ovary and its vascular pedicle to the neck for many years as an experimental model in order to allow greater access to the ovary and its vasculature (Goding et al., 1967; Campbell et al., 1999, 2007). Over 70% of these fresh autotransplants exhibit immediate restoration of ovarian function (B.K. Campbell, unpublished observations), have normal gonadotrophin concentrations and remain functional for more than 10 years (Souza et al., 1998). The aim of this study was to utilize this established surgical model in order to assess the effect of cryopreservation and perfusion on both ovarian function, in terms of follicular development, and endocrine status of the animal, and on vascular tissue damage and patency over a period of several months.

During June and August 2006, under general anaesthesia, all ewes underwent a midline laparotomy and exposure of the reproductive tract. All ewes had their left ovary and associated vascular pedicle dissected free of surrounding tissue back to the origins of the ovarian artery and vein at the dorsal aorta and vena cava, respectively. Care was taken to minimize trauma to surrounding tissues to reduce the risk of adhesion formation.

Prior to tissue removal, heparin (5000 IU ‘Multiparin’ 25 000 IU ml⁻¹ heparin sodium, CP Pharmaceuticals Ltd, Wrexham, UK) was given to reduce any thrombosis risk and 10 ml lignocaine (‘Lignol’ Arnolds Veterinary Products Ltd, Shropshire, UK) was applied over the ovarian pedicle to dilate the artery in preparation for cannulation. The ovarian vein was ligated at the vena cava and cut close to it at a right angle. The aorta was partially occluded using a paediatric Derra clamp around the origin of the ovarian artery which was then carefully dissected together with a small patch of aortic tissue surrounding the origin (Goding et al., 1967). At this point the ovary and pedicle section were ready for cannulation and perfusion (see details below). The aorta was then repaired with complete haemostasis.

In those animals where the ovary was to be re-grafted to the neck (control n = 4; cryopreserved n = 4), the right ovary was ligated, removed at the hilus and fixed in Bouin’s fixative. The right ovary was then carefully dissected together with a small patch of aortic tissue surrounding the origin (Goding et al., 1967). At this point the ovary and pedicle section were ready for cannulation and perfusion (see details below). The aorta was then repaired with complete haemostasis.

‘Cryopreserved’ group

Whole ovarian cannulation and cryopreservation

The cryopreservation media was based on that used previously for WOCP (Onions et al., 2008): Leibovitz L-15 media supplemented with 1.5 mol l⁻¹ dimethyl sulphoxide (DMSO), 0.1 mol l⁻¹ sucrose and 10% (v/v) heat inactivated fetal calf serum (FCS). The media was adjusted to pH 7.3–7.4 and filter sterilized using a 0.22 nm syringe filter (Minisart®, Sartorius biotech, Surrey, UK) prior to use.

Following removal of the ovary and pedicle from the ewe, the ovarian artery was cannulated through the origin using a 2F (0.7 mm OD) or 2.5F (0.75 mm OD) intravenous cannula (Sims Portex Ltd, Kent, UK) and tied into place using a 0 mersilk non-absorbable suture. The ovary was then placed in a sterile perfusion tray and immersed in cryopreservation media. The ovary was initially perfused, via the cannula, with cold, 2.5F (0.75 mm OD) intravenous cannula (Sims Portex Ltd, Kent, UK) fitted with a 50 ml syringe. All solutions were refrigerated at an increased rate of 0.2°C min⁻¹ before being perfused with the cryopreservation media for 60 min at a rate of 0.5 ml min⁻¹ using a syringe driven perfusion pump (Precidor Infors Ag Basel; ChemLab Scientific Products Ltd, Hornchurch, UK) fitted with a 50 ml syringe. All solutions were refrigerated before use and all perfusions were carried out on ice.

Each ovary and cannulated pedicle was placed in a 15 ml cryogenic vial (Scientific Laboratory Supplies, Nottingham, UK) and covered with cryopreservation media. A slow-freezing cryopreservation protocol was utilized which involved cooling to −9°C at −1°C min⁻¹ before manual seeding was carried out. The temperature was then reduced at −0.2°C min⁻¹ to −40°C following which the temperature was lowered at an increased rate of −10°C min⁻¹ to a final temperature of −140°C. The cryogenic vials were then plunged into and stored in liquid nitrogen for 4–5.5 weeks until autotransplantation.

Ovarian thawing

Each ovary was rapidly thawed in a 37°C water bath as described previously (Onions et al., 2008) and transferred to a laminar flow hood. The ovary and pedicle were placed in a perfusion tray and immersed in and perfused with warm thawing media. Three thawing media were...
used consisting of Leibovitz L-15 media supplemented with 10% FCS and reducing concentrations of DMSO (1, 0.5, 0 M). Sucrose was included only in the first thawing media at 0.1 M. All three thawing media (pH 7.3–7.4) were filter sterilized prior to use and perfused warm (37°C) through the ovary for 10 min at a rate of 1 ml min⁻¹. The cannula was then eased from the ovarian artery ready for reanastomosis.

Ovarian reanastomosis to the carotid artery and jugular vein was carried out as described previously by Goding et al. (1967). Briefly, the carotid artery and jugular vein were exposed by blunt dissection taking care to avoid the vagus nerve. By means of the aortic patch, the ovarian artery was reanastomosed to the carotid artery with approximately 6–8 individual stitches of 6/0 prolene monofilament. Immediate arterial patency was confirmed visually. A side-to-end reanastomosis was then performed between the jugular and ovarian veins using a continuous suture of 6/0 prolene monofilament. The ovary was then loosely sutured in position in a pocket created under the skin of the neck using between three and four stitches of 0 mersilk (Fig. 1). A sterile drain was loosely inserted to allow post-operative drainage of the site for 48–60 h as required.

Control group
Following removal, the left ovary was transferred to a laminar flow hood and the ovarian artery was cannulated as described above. The tissue was immersed in a cold perfusion media consisting of Leibovitz L-15 media supplemented with 10% (v/v) FCS (pH 7.3–7.4 and filter sterilized). The ovary was initially perfused with cold, heparinised (100 IU ml⁻¹) Ringer’s solution for 10 min at a rate of 1 ml min⁻¹ followed by the cold perfusion media at 0.5 ml min⁻¹ for the length of time required for exposure of the graft site; typically 20–30 min. The perfusions were carried out on ice. The neck site was prepared and the ovarian artery and vein were reanastomosed to the carotid artery and jugular vein, respectively, as described above.

Post-surgery
Following ovarian removal, twice weekly jugular venous blood samples were collected from all ewes into heparinised vacutainers (170 IU Lithium heparin vacutainer, BD Vacutainer Systems, Plymouth, UK). This was increased to daily samples for the first 7 days after re-grafting surgery, following which, twice weekly collections were resumed. Plasma was obtained and stored at −20°C until analysis for FSH, inhibin A, LH and progesterone concentrations.

Starting 1–5 weeks after ovarian autotransplantation, weekly transdermal scanning of the ovary and pedicle was carried out using a 7.5 MHz scanning probe and monitor (Aloka SSD 500, Switzerland).

In February 2007, 7–8 months post-transplant, luteal regression was induced by administration of 125 mg cloprostenol (Estrumate™, Schering-Plough Animal Health, Welwyn Garden City, UK), following which oestrus was detected by marking of the ewe by a raddled ram. Then 13–17 days later, the ewes were euthanised and the transplanted ovary and pedicle were exposed. Initial visual observations of the tissue were made, including ovary size, the presence of visible antral follicles and corpora lutea (CLs) and the general appearance of the pedicle. Patency of the ovarian vascular supply was assessed by exposing the carotid artery and occluding either side of the anastomosis site to ensure flow only to the ovary. Saline was perfused slowly into the carotid artery to flush any residual blood, followed by trypan blue dye (0.4%) to give a clear visualization of flow through the ovarian artery and vein (Fig. 2). The ovarian tissue and pedicle were then dissected out and fixed separately in Bouin’s fixative before closure of the abdomen.

Blood plasma hormone analysis
Plasma FSH concentrations were measured using a direct radioimmunoassay based on the method of Campbell et al. (1990) using iodination grade oFSH (tert.ofSH/igl Tucker Endocrine Research Institute, LLC, Tucker, Georgia, USA) and oFSH antiserum (rabbit; NIDDK-anti-oFSH-1; AFP-CS288113). Assay sensitivity was 0.16 ng ml⁻¹ with a coefficient of variation (CV) of <15%.

Plasma LH concentrations were measured using a direct radioimmunoassay based on the method of Mann and Lamming (2000) using iodination grade oLH (NIDDK-oLH-I-2) and oLH antiserum (NIDDK-anti-oLH R16). Assay sensitivity was 0.2 ng ml⁻¹ with a CV of <12%.

Plasma progesterone concentrations were also measured using a direct radioimmunoassay (McNeilly et al., 1986) using radiolabelled progesterone, 11α-Progesterone Glucuronide-Tyramine I125 (Amersham, GE Healthcare UK Ltd, Buckinghamshire, UK), and an anti-progesterone antibody (SAPU R7044X). Assay sensitivity was 0.06 ng ml⁻¹ with a CV of <18%.

Plasma inhibin A levels were measured by a two-site ELISA as described previously (McNeilly et al., 2002) using a dimeric inhibin βA subunit monoclonal capture antibody (E4 clone) and a αC-specific biotinylated monoclonal antibody. Assay sensitivity was 32 pg ml⁻¹.

Tissue analyses
Serial sections (5 μm) were made of both non-grafted (right) ovaries and recovered grafted (left) ovarian tissues from all ewes. Sections of ovarian
tissue 100 μm apart (n = 5) were stained with haematoxylin and eosin (Brown, 1969). Within the ovarian cortex of these sections, 20 consecutive fields of view were studied under magnification (×400) and 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).

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

Results

A summary of the treatment and type of ovariectomy for each ewe along with the immediate post-surgical findings are given in Table I. The Planer freezer suffered a malfunction during the freezing programme for one cryopreserved ovary (from ewe 726) which had to be plunged directly into liquid nitrogen from −30°C. This animal subsequently showed no evidence of ovarian function and therefore data generated from ewe 726 was excluded from the hormone profile analyses.

Immediate vascular patency

Immediate vascular patency was observed in all grafts following arterial reanastomosis. In two frozen-thawed ovaries, however, evidence of localized microvascular haemorrhages were observed on the pedicle following reanastomosis (Fig. 3).

Table I Table summarizing the treatment groups used in this study

<table>
<thead>
<tr>
<th>Ewe ID</th>
<th>Treatment/Control Ovariectomy</th>
<th>Surgery Notes</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>720</td>
<td>Cryopreserved</td>
<td>Hemi-ovariectomy</td>
</tr>
<tr>
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<td>Hemi-ovariectomy</td>
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</tr>
<tr>
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<td>Cryopreserved</td>
<td>Bilateral</td>
</tr>
<tr>
<td>725</td>
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<td>Cryopreserved</td>
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<tr>
<td>727</td>
<td>Cryopreserved</td>
<td>Hemi-ovariectomy</td>
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<tr>
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<tr>
<td>729</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>730</td>
<td>Cryopreserved</td>
<td>Hemi-ovariectomy</td>
</tr>
</tbody>
</table>

The type of ovariectomy performed during the initial surgery on each of the treated animals is also detailed along with any notes arising from the immediate post-surgical period.

Hormone profiles

For analysis of the hormone profiles generated, ewes were initially grouped according to their treatment group (cryopreserved or control). Ewes in the ‘cryopreserved’ group were then further categorized according to their FSH profile into either ‘mid-castrate’ or ‘castrate’ groups. Although some ewes showed a decline in FSH levels some months after autotransplantation into the mid-castrate range (2–8 ng ml⁻¹; Baird et al., 1999) and were therefore classified as the ‘mid-castrate’ group for the subsequent hormone profile analysis, the remaining ewes exhibited castrate range FSH concentrations throughout the study and were classified as the ‘castrate’ group. Mean data was calculated for the hormone profiles and a summary of the FSH, inhibin A, LH and progesterone results for the three groups are illustrated in Fig. 4. Representative profiles from animals in each group are also shown in Figs 5–7.
In the cryopreserved group, three ewes achieved mid-castrate FSH levels following autotransplantation of cryopreserved ovaries (Fig. 4A). Overall, FSH levels increased to castrate (>8 ng ml$^{-1}$) levels by 1 week after autotransplantation and remained in this range for approximately 5 months after surgery (range 3.5–5.5 months). FSH concentrations then declined to mid-castrate levels (2–6 ng ml$^{-1}$) for the remainder of the study, or at least until luteal regression was induced (Fig. 5). All the ewes in this group were initially intended for orthotopic autotransplantation and accordingly were only hemiovariectomised at the initial surgery. These animals exhibited a marked increase in inhibin A levels following hemi-ovariectomy (both on average and individually; Figs 4A and 5) but following ovarian autotransplantation and removal of the right ovary, inhibin A was undetectable (<32 pg ml$^{-1}$) for approximately 4 months before increasing to approximately 100 pg ml$^{-1}$ at the time of euthanasia (Fig. 4A). LH levels remained low (<1 ng ml$^{-1}$) in the time between ovary removal and reanastomosis (hemi-ovariectomy period) but increased to 1–4 ng ml$^{-1}$ following ovarian autotransplantation for 3 months (Fig. 4B). By 4.5 months, however, levels had declined to 1 ng ml$^{-1}$ or less and remained at this concentration for the rest of the study. Evidence of an LH surge was seen in one ewe (730) 3.5 days after PGF2α administration, with levels rising transiently to over 22 ng ml$^{-1}$, however, the progesterone profile did not provide evidence of any subsequent functional CL formation. One ewe in this group, however, did display elevated plasma progesterone levels, increasing 23.5 weeks after whole ovarian autotransplantation (Fig. 5). The display of oestrous behaviour and observation of a CL during transdermal scanning confirmed ovulation. Progesterone levels remained high until PGF2α administration when levels fell transiently before increasing again 8 days later.

Of the four cryopreserved group ewes which fell into the castrate group, three were bilaterally ovariectomised during ovary removal surgery. FSH concentrations increased rapidly following surgery to castrate levels (>10 ng ml$^{-1}$) and remained at this concentration following ovarian autotransplantation and for the remainder of the trial (Fig. 4C). LH levels were also elevated to ~2–4 ng ml$^{-1}$ for the duration of the trial (Fig. 4D). Mean inhibin A levels did not change following ovarian removal and re-grafting, remaining around the 200–300 pg ml$^{-1}$ range for the whole study (Fig. 4C). Individually however, whilst inhibin concentrations in ewe 719 were relatively stable following ovarian removal and transplantation, levels increased to 600–700 pg ml$^{-1}$ approximately 4 months after ovarian thawing and autotransplantation (Fig. 6). This coincided with a period of apparent ovarian activity during which time the ewe displayed oestrous and CLs were evident following transdermal scanning of the ovary. In addition, elevated progesterone levels were observed, indicative of CL function, 22 weeks after receiving a cryopreserved whole ovary graft (Fig. 6). Progesterone levels remained elevated for only 3.5 weeks before falling to low levels. Following PGF2α administration progesterone levels increased only slightly, however, despite this, a CL was seen during post-mortem dissection of the ovary graft.

In the control group, plasma FSH levels rose quickly after surgery, but remained in the mid-castrate range (5–7 ng ml$^{-1}$; Fig. 4E). One ewe (722) demonstrated mid-castrate plasma FSH (3–6 ng ml$^{-1}$) for 6 months following graft surgery, after which time FSH levels increased to castrate levels (>8 ng ml$^{-1}$). Inhibin A levels were relatively low, but detectable (<50 pg ml$^{-1}$) for 4–5 months following autotransplantation after which time concentrations increased slightly, however, a high degree of variation was seen between ewes (Fig. 4E). Plasma LH levels increased after surgery to 2–3 ng ml$^{-1}$, but then began to decline to 1–2 ng ml$^{-1}$ approximately 2.5–3 months after surgery and remained in this range for approximately 3 months before increasing (Fig. 4F). Individually, one ewe (728) displayed evidence of an LH surge, 3.5 days after PGF2α administration, with levels rising transiently to over 20 ng ml$^{-1}$. LH concentrations in another ewe (729) were highly variable until 3.5 months after surgery at which time concentrations consistently fell to below the limits of detection. This coincided with high levels of progesterone 12 weeks after grafting which remained elevated for approximately 17 weeks, indicative of CL formation and function (Fig. 7). This was supported by transdermal scanning images showing the presence of a CL. Levels in this ewe increased again transiently 2 weeks later before administration of PGF2α. Eight days after PGF2α administration, progesterone levels again increased and during post-mortem examination a CL was seen on the transplanted ovary.

**Transdermal scanning**

In five cryopreserved and three control group ewes, the ovarian tissue visibly regressed over the course of the study. In two ewes from the cryopreserved group (719 and 727) and one control ewe (729), a significant amount of ovarian tissue could be seen for the duration of the scanning period. A CL was first evident in the ovarian tissue of the control ewe 18 weeks following autograft surgery, although hormone profiles suggest it may have been present up to 2 weeks earlier (Fig. 7). The first CL in a previously cryopreserved ovary (ewe 727) was observed 25 weeks after transplantation and its function was confirmed by blood hormone analyses (Fig. 5). In the second ewe from the cryopreserved group (719), the ovary could not be visualized for over 5 months. However, the ewe then displayed oestrus and evidence of CL formation and subsequent luteal regression was confirmed by scanning and progesterone profiles (Fig. 6). Small CL’s were observed in one cryopreserved (730) and one control group ewe (725) 25 and 28 weeks post-grafting, respectively, but their presence/function was not supported by the progesterone profiles.

**Long-term vascular patency**

Following euthanasia of the ewes, perfusion of dye confirmed fully patent ovarian vasculature in 10 out of the 12 transplants (Fig. 2).
Two ewes had non-patent ovarian arteries and one of these was ewe 726 for whom the Planer freezer malfunctioned during the freezing programme. In the other ewe, (728; control) dye was only seen to perfuse approximately half way up the artery before a blockage occurred.

**Follicle counts**

The follicle counts recorded are shown in Table II. There was no significant difference in the density of any type of follicle or total follicle count between the control and cryopreserved groups in the right, non-grafted ovaries confirming that all the ewes were comparable in this respect at the start of the study.

The density of primordial, transitional and primary follicles was significantly reduced in autotransplanted ovaries ($P < 0.001$). Total follicle population also declined significantly, by over 90% in all groups. The extent of primordial and transitional follicle loss was not, however, significantly affected by cryopreservation, being similar in both control and cryopreserved ovaries.

**Figure 4** Mean hormone profiles ($\pm$ SEM) generated for plasma concentrations of FSH, Inhibin A (A, C, E: filled diamond, FSH; filled circle, Inhibin A), LH and progesterone (B, D, F: open diamond, LH; open circle, Progesterone), grouped according to treatment group and FSH profile: (i) 'Cryopreserved' Mid-castrate (ewes receiving cryopreserved ovarian autotransplant with mid-castrate FSH profile restored; graphs A and B), (ii) 'Cryopreserved' Castrate (ewes receiving cryopreserved ovarian autotransplant with castrate FSH profile maintained; graphs C and D), (iii) Control (ewes receiving fresh ovarian autotransplant; graphs E and F).

The black dashed line indicates the time of graft surgery. Note the different scale for progesterone concentrations in F.
Although neither secondary, pre-antral nor antral follicle populations were significantly altered following ovarian autotransplantation in either cryopreserved or control groups, there was a tendency for secondary and pre-antral follicle density to increase in the control group and fall in the cryopreserved group.

Comparing the cryopreserved group ewes with mid-castrate and castrate FSH profiles, although both primordial and transitional follicle numbers were significantly lower following autotransplantation for both groups (Table II), in those ewes where mid-castrate FSH levels were restored at least some, albeit small numbers of follicles were seen to remain compared with a total depletion of follicles in the castrate group. Further, significantly more primary follicles remained in the recovered transplanted ovaries of the mid-castrate than in the castrate group. In addition, although the antral follicle population in the castrate group suffered a non-significant decline over the transplant period, numbers in the mid-castrate group increased significantly suggesting follicle development had resumed in this group. Finally, 7 months post-transplant the total follicle count was significantly reduced in both mid-castrate and castrate profile ewes, however, total follicle count was significantly ($P = 0.057$) higher in the mid-castrate group compared with the castrate group.

**Discussion**

The results of this study show that WOCP and autotransplantation is capable of successfully restoring ovarian function, including the ability to ovulate and form functional CLs, but remains associated with high rates of follicle loss which prevent restoration of full and long-term ovarian function. Importantly, this study has also provided strong evidence that this lack of long-term success is not due to the loss of vascular patency within the ovarian pedicle leading to ischaemia (Bedaiwy et al., 2003), but this explanation has been potentially confounded by the technical difficulty of this procedure and a lack of control data showing high success rates with this approach in fresh autotransplants.

In the current experiment, by excising the ovarian artery at its origin at the dorsal aorta and autotransplanting to the neck, we have utilized a model system in which a much more robust arterial reanastomosis can
Table II: Table showing average follicle densities (±SEM) observed in the cortex of whole ovaries taken either at the initial surgery (Right; i.e. before any treatment) or 7 months after grafting (Left).

<table>
<thead>
<tr>
<th>ID</th>
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<th>Primary</th>
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<td></td>
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</tbody>
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Results are from ewes which received either fresh (Control) or cryopreserved ovarian grafts (Cryo—all). The treated group was then further analysed according to whether the ovary was classified as either primordial, transitional, primary, secondary, pre-antral or antral (Lundy et al., 1999; see Section 2.1.6.2). Different letters represent a significant difference (P < 0.05) either between treatment groups or between right and left ovaries.
onwards (McNatty et al., 1999). Circulating inhibin A levels therefore provide an index of both the pre-antral and antral growing follicle population, and also control FSH release from the adenohypophysis (Baird et al., 1991; Mann et al., 1992).

Results from both the follicle counts and hormone profiles showed that after 7 months, the ovaries autotransplanted following cryopreservation had suffered either a partial or total depletion of follicle population. FSH levels in both groups increased to or were maintained at castrate levels immediately after autotransplant surgery whilst inhibin A levels declined to often undetectable levels. This is common with ovarian autografting and illustrates the loss of growing follicles and disruption to folliculogenesis and ovarian function in the immediate post-graft period (Gosden et al., 1994; Baird et al., 1999; Campbell et al., 2000; Imhof et al., 2006).

The length of time taken for FSH and inhibin A to return to normal levels represents the time needed for surviving primordial follicles within the transplanted ovary to develop to antral stages. In three ewes receiving cryopreserved ovaries, plasma inhibin A levels were restored and FSH concentrations reached a level mid-way between intact and castrate by 3.5–5 months after grafting. Baird et al. (1999) also found that FSH levels only returned to mid-castrate levels (4–5 ng ml⁻¹) at best following autografting of cryopreserved ovarian cortical strips and noted this was indicative of the depletion, but not total loss, of small, inhibin A-producing antral follicles. The ewes in this ‘mid-castrate’ group did suffer significant follicle losses, particularly the primordial to primary stages, which would limit the number able to progress to antral stages, and therefore may explain the incomplete restoration of FSH levels. However, follicle numbers were not totally depleted and in addition, antral follicle numbers in the mid-castrate ewes increased significantly over the transplant period, suggesting some resumption of follicular development to antral stages and supporting the hormonal evidence of some partial restoration of the pituitary-gonadal feedback axis.

Both hormone profiles (particularly FSH levels) and follicle counts in the remaining ewes were consistent with the large scale, if not total loss of ovarian follicles. Previous studies have attributed these large follicle losses and failure of long-term graft/autotransplant success to a combination of cryopreservation and ischaemic damage (Baird et al., 1999; Callejo et al., 2001; Bedaiwy et al., 2003; Salle et al., 2003). We have shown here, however, that follicle losses still occurred despite immediate and continued vascular patency. Thus, other factors related to either the perfusion or cryopreservation protocols must be contributing to this follicle loss. Recent research conducted in our laboratories, however, showed that cryopreservation and thawing of whole ovine ovaries, using a slightly modified version of the protocol developed for cortical autografts, does not significantly affect acute follicle viability in vitro (Onions et al., 2008). These results lead us to speculate that although in-vitro measures of follicle and tissue viability may accurately reflect the current status of frozen tissue post-thaw, they may fail to accurately predict long-term survival in vivo.

The cause(s) of follicle loss following WOCP remains unknown, but an unexpected result from the current study was that the control ewes which received fresh, perfused ovaries suffered similar follicle losses and endocrine disruption as the ewes receiving frozen-thawed ovaries. This finding was highly unexpected as this surgical preparation, as described above, has been routinely performed to prepare ewes with ovarian autotransplants with high success rates for many years and the only difference between the procedures was that the ovarian pedicle was cannulated and perfused with Leibovitz L-15 media supplemented with 10% (v/v) FCS in the controls in the present study. This observation would therefore tend to suggest that factor(s) associated with the cannulation and perfusion of the ovarian pedicle, in addition to cryopreservation, make a significant contribution to the loss of follicles observed following WOCP. This possibility is supported by the observation that ovarian perfusion can disrupt the endothelial cell layer of the vasculature within the ovarian medulla (V.J.O. and B.K.C. unpublished observations).

A further unexpected result from the current study was that all three ewes in the mid-castrate group were initially hemi-ovariectomized, whereas three out of the four castrate ewes whose ovaries failed to restore function were bilaterally ovariectomized at the initial surgery. This suggests that there may be some advantage, in terms of subsequent ovarian function and the restoration of gonadotrophin levels, to hemi-ovariectomy. It is well established that hemi-ovariectomy results in a transient increase in circulating FSH concentrations which in turn stimulates compensatory hypertrophy in the remaining ovary (Findlay and Cumming, 1977; Campbell 1988) and which appeared to be associated with a rapid increase in inhibin A levels in these ewes prior to transplantation (Fig. 4A). As a result of these homeostatic mechanisms, at the time of ovarian autotransplantation, hemi-ovariectomized ewes had maintained intact gonadotrophin concentrations whereas bilaterally ovariectomized ewes had elevated levels. A study in mice found that supplementation with gonadotrophins before and after isografting of whole ovaries significantly increased follicle survival (Imthurn et al., 1999) and concluded that patients should be treated with gonadotrophins prior to ovarian tissue removal. Our results, however, tend to suggest that there is an adverse effect of re-grafting into an environment of high gonadotrophin levels and would tend to advocate the removal of only one ovary for cryopreservation, and transplantation into a low gonadotrophin environment.

In conclusion, these results demonstrate successful partial restoration of ovarian function following cryopreservation of the whole ovary and vascular pedicle in a large monoovulatory species. The inability to restore full ovarian function was related to loss of primordial follicles rather than vascular patency in both frozen and fresh tissue, suggesting that factors associated with cannulation and perfusion may contribute to this depletion. Further work is therefore needed to elucidate these factors before the procedure could be considered a viable option for fertility preservation.

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