Fertility after autologous ovine uterine-tubal-ovarian transplantation by vascular anastomosis to the external iliac vessels

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Background: Transplantation of the uterus has been suggested as a treatment of uterine factor infertility. This study investigates whether the sheep uterus can resume its capacity to harbour normal pregnancies after autotransplantation by vascular anastomosis.

Methods: From 14 ewes, the uterus, excluding one uterine horn, was isolated along with its oviduct and ovary and preserved ex vivo and then transplanted back with end-to-side anastomosis of the vessels of the graft to the external iliac vessels. After recovery, the ewes underwent surgical examination and serum progesterone measurements to ascertain healing and ovarian activity. Afterwards, five autotransplanted and five control ewes were placed with a ram for mating. Caesarean sections were performed before the estimated term of pregnancy and data on fetal measures were compared.

Results: Of the 14 ewes, seven survived surgery with ovarian activity intact and grafts showing normal appearance. Mating occurred in four of five transplanted ewes and in five out of five controls, and three transplanted animals and five control animals conceived. In one transplanted ewe, torsion of the uterus was observed after spontaneous initiation of labour. Foeti from transplanted mothers were comparable in size to those of controls.

Conclusions: Despite the encountered complications, this is the first report to demonstrate fertility and pregnancies going to term after autotransplantation of the uterus in an animal of a comparable size to the human.

Key words: pregnancy / sheep / uterus / infertility / uterine transplantation

Introduction

Uterine infertility exists due to congenital uterine agenesis or after hysterectomy and may also occur in women with congenital uterine malformations, intrauterine adhesions, radiation-damage to the uterus or large/distorting myomas. No population-based studies have been performed to estimate the prevalence of uterine infertility, but approximations indicate that this group of women could consist of as many as 15 000 in the UK (Sieunarine et al., 2005) and 7 million in the USA (Nair et al., 2008). Many of these women have functioning ovaries, but for obvious reasons standard assisted reproductive techniques cannot restore their fertility. Although parenthood can be attained through adoption or, in some countries, IVF surrogacy, infertility can substantially decrease their quality of life (Cousineau and Domar, 2007) and therefore research with the objective of investigating potential treatments for this type of infertility is required.

When the first human uterine transplantation (UTx) was performed in the year 2000 (Fageeh et al., 2002), it was an attempt to introduce UTx as a curative treatment of uterine factor infertility. Although initially successful, complications occurred and the uterus had to be removed due to massive necrosis 3 months after the transplantation (Fageeh et al., 2002). The case identified the need for further preparatory studies and several animal models have now been presented (Brannstrom and Wranning, 2008).

Our group has demonstrated pregnancies with normal offspring after syngenic UTx in mice (Racho El-Akouri et al., 2003a). Moreover, studies on tolerance for cold ischaemia (Racho El-Akouri et al., 2003b) and initial studies on the effects of immunosuppressant drugs on
rejection of the transplanted uterus have been conducted in the mouse model (Wranning et al., 2007). Recently, rat models for uterus transplantation have also been developed by us (Wranning et al., 2008a) and others (Jiga et al., 2003).

Although these small animal models are suitable for detailed studies of the function of the transplanted uterus, development of new surgical methods requires animal models of a larger size. The pig was initially tested in experiments involving autologous UTx (Sieunarine et al., 2005; Wranning et al., 2006). The low success rate in the pig (Wranning et al. 2006) led to development of a sheep UTx model. The methodology in the sheep was based on studies of heterotopic transplantation of ovarian—tubal—uterine specimens with vascular anastomosis to the carotid artery and the jugular vein (McCranken et al., 1971). In studies involving autologous UTx to an orthotopic site, but with vascular connection to the external iliac vessels (Dahm-Kahler et al., 2008; Wranning et al., 2008b) or to the uterine vessels (Ramirez et al., 2008), the feasibility of the technique was demonstrated.

The obvious next step in UTx research in the sheep is to assess long-term function and fertility potential after autotransplantation. This would assess whether the surgery and a new vascular supply of the uterus after transplantation would affect function. In the present study, we demonstrate, for the first time, pregnancies after UTx in a large animal model.

Materials and Methods

Study design

All ewes were hormonally synchronized as previously described (Wallin et al., 2009) to be at the same pre-ovulatory stage at surgery for standardization. After autotransplantation of the uterus with the right oviduct and ovary, the ewes recovered for 3–8 weeks before assessment of surgical success was performed by inspection of the vaginal anastomosis and cervix as well as by measurement of serum progesterone levels. Moreover, two ewes were laparotomized at this time point to ascertain normal appearance and contractions of the uterus. Five transplanted ewes were introduced to a ram for the duration of four oestrus cycles. Five non-transplanted ewes of the same breed and age were included in the herd to serve as controls. Mating was monitored and pregnancies were confirmed at the beginning of the third trimester by general assessment of mammary and body growth as well as by abdominal ultrasound. At a predecided date during the third trimester, a Caesarean section was performed and the uterus and foeti were examined before the ewe and lamb/-s were euthanized.

Animals

Mature ewes (2–5 years of age; 50–70 kg) of Swedish pelt sheep breed (Gotland sheep, n = 14) of proven fertility were purchased from an accredited breeder. The animals arrived at the surgical facility 3 weeks before the experiments and were kept in a controlled light/dark cycles of 12 h/12 h. The animals had free access to hay and water and were fed pelleted concentrated fodder twice a day. The study followed the guidelines for the handling and care of experimental animals issued by the Swedish Federal Animal care Agency and was approved by the Animal Ethics Committee in Gothenburg, Sweden as well as the Institutional Review Board.

Anaesthesia and pain-relief

At 24 h prior to surgery food was withdrawn and carprofen (Rimadyl®; Pfizer, New York, NY, USA; 2.0 mg/kg, i.m.) was given to induce analgesia. In addition to our established protocol for anaesthesia of sheep (Dahm-Kahler et al., 2008) peri- and post-operative epidural analgesia was also used. This was accomplished by inserting an 18G epidural catheter (Perifix®; Braun Medical Inc., Bethlehem, PA, USA) into the sacral epidural space with advancement 10–15 cm cranially. Epidural morphine (Meda AB, Solna, Sweden; 0.1 mg/kg initial dose followed by 0.05 mg/kg every 6–12 h) was given during and for 3 days after surgery. In addition, carprofen (Rimadyl®; Pfizer, New York, NY, USA; 2.0 mg/kg/day, i.m.) was given once daily for 7 days and at signs of severe pain buprenorphine (Temgesic®; Schering-Plough, Kenilworth, NY, USA; 0.012–0.024 mg/kg, i.m.) was given.

Surgery

The objective of surgery was to isolate a specimen including the common uterine cavity and cervix, the left uterine horn and the ipsilateral ovary and oviduct. The right uterine horn and adnexae were to be surgically removed and the ovary of that side was used for ovarian cryopreservation experiments (Wallin et al., 2009). The surgical technique used in the present study follows that previously published for autologous UTx in the sheep (Dahm-Kahler et al., 2008) but with some modifications that include the ovary and oviduct. The main surgical procedure is only briefly outlined below and novel procedures are described in detail.

Through a midline incision, the origins of left internal iliacs and the left ovarian artery were freed, with further vascular dissections in a distal direction to include the left uterine and ovarian vessels. The right uterine horn and adnexae were excised followed by separation of the ureters from the left uterine vessels and the cervix. The upper vagina was transected and at this stage the uterus with the left adnexae was only attached to the animal by a vascular pedicle including the uterine-anterior branch of iliac artery, the utero-ovarian vein and the ovarian artery. Heparin (10 000 IU) was given i.v. to prevent blood clotting before an ellipsoid-shaped patch of the aortic wall, including the origin of the left ovarian artery, was isolated. The left internal iliac artery was clamped 5 mm distally to its branching from the common iliac artery. The posterior branch of the internal iliac artery was clamped around 2 cm distal to its branching and then cut to be cannulated in a retrograde direction.

The organ was flushed in situ with chilled (4°C) Perfadex preservation solution (a gift from Vitrolife, Mölndal, Sweden) under a pressure of 100 mmHg, which is similar to the systolic blood pressure in sheep. The utero-ovarian vein was opened to allow uterine outflow of blood and preservation solution. The durations from heparin injection to organ removal and from Perfadex perfusion until removal were <10 and <5 min, respectively. The utero-tubal-ovarian specimen was then, under continuous flushing, removed and placed ex vivo on sterile saline ice slush for around 1 h. The clamped proximal ends of the left internal iliac artery and the left utero-ovarian veins were ligated. The aortic wall defect was sutured with a 6-0 polypropylene suture. Autotransplantation was achieved by end-to-side anastomosis of the graft vessels to the left external iliacs and the left utero-ovarian veins were ligated. The aortic wall defect was sutured with a 6-0 polypropylene suture. Autotransplantation was achieved by end-to-side anastomosis of the graft vessels to the left external iliacs and the left utero-ovarian veins were ligated. The aortic wall defect was sutured with a 6-0 polypropylene suture. Autotransplantation was achieved by end-to-side anastomosis of the graft vessels to the left external iliacs and the left utero-ovarian veins were ligated. The aortic wall defect was sutured with a 6-0 polypropylene suture. Autotransplantation was achieved by end-to-side anastomosis of the graft vessels to the left external iliacs and the left utero-ovarian veins were ligated.

Initial post-operative recovery was assessed by monitoring blood gas values and general physiological measures such as pulse, diuresis, food and water intake and ability to stand up. Animals that showed satisfactory recovery within the first 24 h were returned to the herd. To prevent post-operative blood clotting, low-molecular-weight heparin (dalteparin-natrium, Fragmin®, Pfizer, Sollentuna, Sweden; 100 mg/kg, s.c.) was given.
Fertility after uterine transplantation

Assessment of graft viability

In all ewes, immediate surgical outcome was assessed 30–60 min after reflow by inspection of the patency of the vascular anastomosis sites, pulsation through the transplanted vessels, colour of the transplant and occurrence of spontaneous uterine contractions. To evaluate long-term outcome, ewes showing satisfactory post-operative recovery were examined 3–8 weeks after surgery. Ovarian cyclicity was verified by measurement of serum progesterone and uterine/vaginal healing was assessed either by laparotomy (first two animals) or only by vaginal examination (remaining five animals). These blinded examinations were part of a protocol that would assure satisfactory healing to enable mating.

During laparotomy, the transplanted organs and the anastomosis sites were inspected. Uterine contractility (spontaneous and oxytocin-induced) was measured as previously described (Dahm-Kahler et al., 2008). The healing of the vaginal anastomosis was assessed by manual distension of the vaginal–vaginal anastomosis site and incisions to detect tissue blood flow were made into the upper vagina, vagina, myometrium and ovary. The extent of blood flow over the arterial anastomosis sites was evaluated by severance of the vein followed by the two anastomosed arteries.

The vaginal anastomosis sites of the animals selected for evaluation of fertility (n = 5) were examined under light anaesthesia 6 weeks post-operatively, using a plastic laparoscopic trocar inserted into the vagina with insufflation of air through the gas channel.

Analysis of serum progesterone

Serum progesterone was measured by the use of a progesterone analysis kit and an automated procedure (Architect i1000; Abbott Laboratories, Abbott Park, IL, USA). In two ewes, blood samples were taken every third day over 18 days. Serum progesterone was analysed daily and if no elevated levels were seen after a week, a single injection of 500 IU of equine chorionic gonadotrophin (eCG; Folligon™; Intervet Inc, Millsboro, DE, USA) was given. In the five remaining ewes synchronization of cycle phase was performed as described in the preoperative treatment section and serum progesterone was measured on Day 0, 3 and 5 after eCG administration.

Assessment of fertility

There were five transplanted and five untreated control animals returned to the breeder for mating. After 2 weeks, a ram of proven fertility was introduced into the herd. The ram wore a chest harness containing coloured dye. When the ram mounts, gains intromission and ejaculates, his position and behaviour will result in a distinct mark on the ewe’s back that is distinguishable from diffuse markings around the hind-quarters resulting from false mounts. Markings indicative of mating (hereafter referred to as mating) were checked every morning and the colour of the dye was changed every week to detect repeated mating. The ram was kept with the ewes for the duration of four cycles (60 days). At the end of the second trimester (90–100 days, calculated from the day of first recorded mating), all ewes returned to the research facilities and were inspected concerning abdominal contour/mammary growth and with transabdominal ultrasound. The examiners were blinded to the identity of the animal.

On estimated pregnancy Day 130–142 (of 145 days average pregnancy duration in sheep), transplanted and control animals underwent a planned Caesarean section under isoflurane anaesthesia. Lambs were checked for vital signs (heartbeat, chest movements) and then euthanized. Placental, body and organ weights as well as crown-rump lengths were recorded. In the ewes, the uterus and ovary as well as the vascular anastomosis sites were inspected and biopsies were taken from the uterus and ovary. The ewes were euthanized by i.v. injection of 40 ml saturated KCl solution whereas under deep anaesthesia.

Results

Outcome of surgery

The median duration of surgery from incision to closure was 522 min (range 420–620 min) and the estimated blood loss was <25 ml in all animals. In all 14 autotransplanted sheep, recirculation of the graft was satisfactory according to colour change from white to reddish, pulsation through the anastomosed vessels and spontaneous uterine contractions when inspected 30–60 min after transplantation. In some animals (n = 3) uterine contractions were recorded and contraction curves prior to (right uterine horn) and 30 min after transplantation (left horn) were found to be similar (Fig. 2).

Of the 14 autotransplanted ewes, seven were excluded from further experiments due to partial paralysis of one hind leg (n = 3; animal numbers 4, 7, 11; Day 1–2 post-operatively), ileus (n = 2; animal numbers 9, 13; Day 2 and 10), peritonitis (n = 1; animal number 2;...
Assessment of surgical outcome 2–4 months post-transplantation

Of the seven remaining ewes, two were examined in more detail 2 months after surgery, to ascertain that the procedure had not caused changes that would risk the animals’ well-being during the fertility experiments, as required by the university veterinarian.

Ovarian function was indicated by spontaneous cyclicity in one ewe and by induced (500 eCG) luteal phase the another (Fig. 3). Subsequent laparotomies showed normal gross morphology of the graft as well as the vascular and vaginal anastomosis sites. Spontaneous and oxytocin-induced uterine contractions were recorded (Fig. 4). The uterus and ovary bled when incised. The vascular anastomosis sites appeared completely healed with a filled utero-ovarian vein and pulsating uterine artery. The vaginal anastomosis site was flexible at distension. Profuse tissue bleeding was also seen after incisions into the cervix and the vagina at a site cranial to the anastomosis line after the uterine vascular anastomoses had been severed.

In the five remaining ewes, vaginal examination by the use of a laparoscope showed pinkish cervical colour and uninterrupted vaginal mucosa over the vaginal anastomosis site (Fig. 5). Vaginal distension by air insufflation revealed no signs of stenosis.

Fertility and offspring

The five ewes intended for mating also underwent tests of ovarian function. After hormonal synchronization and eCG injection (Wallin et al., 2009) progesterone levels began to rise on Day 3 after eCG (from below the detection limit 0.32 to average 3.8 nmol/l ± 1.09 SD), indicative of corpus luteum formation. Within a month of mating experiments, single mating events had occurred in all controls and in three of five transplanted ewes. One transplanted ewe mated a second time on the last day of the 60 day period. In another transplanted ewe, no mating occurred. A summary of the mating results is given in Fig. 6.

Examinations to determine pregnancies were performed at 90–110 days after the first matings. Body contour, mammary growth and abdominal ultrasound indicated pregnancies in all control animals and in three of five transplanted ewes. Two control ewes gave birth to healthy twin female lambs (both on Day 142 of pregnancy) before the scheduled date for Caesarean section. The other control animals underwent Caesarean section on gestational Day 132, 132.
and 139, bearing a triplet, a singleton and a twin pregnancy, respectively. The fetuses were vital with appearance as well as body and organ weights appropriate for gestational age, sibling numbers and sex. One transplanted ewe showed signs of initiation of labour on Day 140 of pregnancy with restless behaviour, regular abdominal contractions and vaginal dilation. Abdominal contractions ceased after 24 h and a Caesarean section was performed. The uterus was found to be rotated 360° at the lower portion and the twin female lambs were dead. Uterine and ovarian gross and microscopic morphologies displayed signs of blood stasis. The lambs showed no signs of maceration and their body and organ weights were normal for gestational age, sibling number and sex.

The three remaining transplanted and mated ewes underwent Caesarean section on estimated gestational Day 88, 138 and 139, bearing a single female lamb, no pregnancy and a single male lamb, respectively. The lambs were appropriately developed for estimated gestational age, sibling number and sex, showing vital signs. Uterine and ovarian gross and histological morphologies were comparable to controls. The non-pregnant ewe had a uterus and ovary of normal appearance and patent vascular anastomoses. Methyleneblue solution was injected into the uterine cavity via the cervix and the dye filled the uterine and tubal cavities half way up through the Fallopian tube, indicating tubal occlusion. The non-mated, transplanted ewe displayed normal uterine gross morphology, but the ovary was covered by adhesions.

The relation between fetal body weight and crown-rump length, heart weight and placental weight were found to be similar in controls and transplanted animals (Fig. 7a–c).

Discussion

To develop UTx into a clinically safe procedure with reasonable chances of a positive outcome many aspects of the procedures involved need to be thoroughly addressed in animal models. It is therefore desirable to use animal models where different aspects can be isolated and studied in appropriate contexts. The sheep has body and organ sizes as well as reproductive features that are more similar to those of a woman when compared with other large experimental animal models such as pigs, dogs and rabbits. In the present study, the objective was to investigate whether surgery and a new vascular supply affects the fertility potential of the transplanted uterus. To isolate these aspects from immunological reactions and effects of immunosuppressant drugs also present at allogeneic transplantation, we used a model for autotransplantation.
In the present model one uterine horn was removed from the graft with the purpose of reducing the number of required vascular anastomoses and thus reducing surgery times. Our previous findings in the mouse (Racho El-Akouri et al., 2002, 2003a) and rat (Wranning et al., 2008a) have shown that one uterine horn can be removed and that unilateral blood supply is sufficient for full function. Furthermore, the ipsilateral ovary and oviduct were included in the graft to allow for conception by mating. In ruminants, luteolysis and thereby cyclicity is dependent on influences on the ovary of uterine-produced prostaglandins (McCracken et al., 1972) which may be transported to the ovary by counter current mechanisms (Bonnin et al., 1999). With the ovary included in the transplant, ovarian cyclicity as indicated by mating and serum progesterone changes would give more information on the quality of the transplantation procedure than a procedure involving embryo transfer (Papadopoulos et al., 2002). Several technical components of the surgery in the present study are standard procedures of transplant surgery such as vascular flushing, cold ischaemic preservation of the transplant and the technique and site used for vascular anastomosis. In addition, and specific for UTx, is the difficulty in gaining surgical access to the deep pelvis, in obtaining sufficiently long vascular pedicles in the donor and in separating the ureters from the organ.

The long-term surgical success in terms of animal survival was only 50% which of course is unacceptably low. However, in five of the seven euthanized ewes, the cause of loss was presumably species-related. The partial hind leg paralysis in three animals may have been due to injury to the obturator nerve caused by the animal’s supine position during prolonged surgery. In these animals sensory response and some muscle control was intact whereas abductor muscle control was absent. In two other animals, feeding and defecation were interrupted within 2–6 days after surgery. Post-mortem analysis revealed swollen intestines with signs of oedema and discolouration of the intestinal walls. Ruminants are susceptible to paralytic ileus (Bueno et al., 1978) and in these two cases, peri-operative intestinal ischaemia caused by over-extensive packing of the intestines may have induced ileus. In later experimental series using this model, these complications have been abolished by adjustments of protocols concerning the peri-operative positioning of the animal and packing of the intestines.

Two additional animals were lost; one due to acute peritonitis caused by an intestinal rift discovered at post-mortem examination and one to bleeding, most likely derived from the anastomosis site. These two animals were the first losses in the series and naturally there is a learning curve for the procedure, as shown in the mouse (Racho El-Akouri et al., 2002) and rat (Wranning et al., 2008a). These results further emphasize the need for surgical training of this difficult procedure in animal models before introduction in the human as an experimental procedure.

The reproductive performance in the five autotransplanted sheep appeared to be inferior to that of the control animals but the causes are not clear since all animals showed ovarian response to hormonal stimulation. However, it can be speculated that trauma and secondary inflammation after surgery may interfere with ovarian function or cause tubal occlusion as demonstrated in one case. The one animal that did not mate also had the longest duration of surgery which might correlate with an increase in post-operative inflammation interfering with ovarian function.

The decision to terminate the experiments just prior to expected deliveries was motivated by a calculated risk of complications at vaginal delivery due to the vaginal anastomosis and altered structural support. In one case, however, labour started but could not proceed until delivery due to torsion of the uterus. The torsion would have occluded the blood flow to and from the uterus and the placental-fetal unit, thereby causing intrauterine death of the lambs. There were no

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**Figure 7** Diagrams showing crown-rump length to body weight (A), heart weight to body weight (B) and placental weight to body weight (C) of lambs at Caesarean section. Gender symbols represent individual lambs and connectors indicate siblings (autotransplanted = filled symbol, control = open symbol).
signs of maceration of the lambs, indicating that fetal death may have taken place after spontaneous initiation of labour. This finding raises concern for a possible future human trial. Even though our suggestion is that delivery after UTx in a woman should be by Caesarean section, the uterus must be provided support that prevents torsion or prolapse whereas allowing for growth during pregnancy.

Earlier reports on utero-tubal-ovarian autotransplantation in the dog reported pregnancies (Eraslan et al., 1966), but in these studies the specimen was not removed from the abdominal cavity and may be regarded as re-anastomosis experiments. In other experiments in the dog (Scott et al., 1970) and in the rhesus monkey (Scott et al., 1971), the uterus was removed from the abdomen by an avascular technique, with neovascularization after omental wrapping. No pregnancies were achieved in these experiments, even though menstruation resumed in the rhesus monkey (Scott et al., 1971). The present study demonstrates thus for the first time that normal pregnancies can be achieved after removal and autotransplantation of the uterus in a larger animal. We conclude that the use of the external iliac vessels as recipients of the graft vessels is a feasible method to secure blood flow. The complications encountered identify issues of concern such as post-operative inflammatory reactions that might interfere with ovarian and uterine function and the need for structural support of the uterus. These issues must be addressed in more detail before human trials could commence.

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