Fertility and immunosuppression in allogeneic uterus transplantation

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International Ph.D. Thesis

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Progress is impossible without change, and those who cannot change their minds cannot change anything.

George Bernard Shaw, Irish playwright

1 CONTENT

2 SPANISH SUMMARY

A pesar de los avances en medicina reproductiva alcanzados durante las últimas tres décadas, la esterilidad absoluta de origen uterino (AUFI, por sus siglas en inglés), a día de hoy, carece de tratamiento. Ésta, puede ser causada tanto por la ausencia del útero (congénita o adquirida) o la no funcionalidad del mismo [1, 2]. Cálculos en el Reino Unido han demostrado que hay alrededor de 12000 a 15000 mujeres en edad reproductiva con infertilidad uterina en dicho país [3]. No hay cálculos correspondientes a España, pero extrapolando datos sobre prevalencia de causas de AUFI, se estima que puede haber alrededor de 2000-3000 mujeres en edad fértil que no tienen ninguna oportunidad de ser biológicamente madres.

Hasta ahora, las únicas opciones para conseguir la maternidad genética en mujeres con AUFI son la adopción o la maternidad subrogada (coloquialmente llamado vientre de alquiler). Esta última permite poder adquirir la maternidad genética, aunque al igual que en la adopción, se debe adquirir la maternidad legal. La subrogación gestacional se permite solo en algunos países y generalmente conlleva otro tipo de consideraciones de tipo éticas, religiosas, sociales y jurídicas en términos de autonomía, pago, de la salud física y psíquica de la mujer gestante [4, 5]. En cualquier caso ésta opción es ilegal en nuestro contexto nacional.

Se ha sugerido que el trasplante de útero (UTx) podría ser una alternativa a la maternidad subrogada para alcanzar maternidad genética. El UTx no sería un tipo de trasplante que estuviese destinado a suplir una función vital, sino que entraría dentro de la categoría de trasplantes que mejoraran la calidad de vida supliendo la función de un órgano faltante, como por ejemplo los trasplantes de

extremidades o la cara [6] ya que el objetivo del UTx es tratar la infertilidad, que esta asociada con una alteración de la calidad de vida [7].

Cuando los experimentos de la presente tesis fueron diseñados, la evidencia disponible respecto a la fertilidad tras trasplante alogénico e inmunosupresión en el contexto del UTx era limitada, no habiendo estudios sobre la viabilidad del procedimiento y por tanto tampoco sobre los efectos que éste podría tener sobre la descendencia.
Igualmente existían en la literatura datos contradictorios sobre el efecto del trasplante en la fertilidad; mientras que algunos estudios encuentran una mejoría de las tasas de embarazo después de un trasplante de órgano sólido, otros encuentran una mayor tasa de aborto espontáneo y de resultados perinatales adversos.

Por tanto, los diferentes estudios presentados en esta tesis tienen como objetivo construir una base de conocimiento pre-clínica sobre la aspectos relacionados con la fertilidad después de UTx que puedan cimentar un posterior ensayo clínico acorde a las recomendaciones del consenso IDEAL [8]. En los siguientes capítulos se describirán seis estudios publicados en 9 manuscritos que valoran el efecto del UTx y distintos regímenes inmunosupresores sobre la ovulación, la implantación, el desarrollo del embarazo y el fenotipo descendencia. Igualmente se presenta un estudio poblacional que utiliza datos epidemiológicos de dos registros completos únicos en el mundo, con más de 2,500,000 de controles y más de 2000 nacimientos de madres trasplantadas, para evaluar el efecto de la inmunosupresión en el resultado del embarazo de pacientes trasplantadas de órganos sólidos; y finalmente, esta tesis describe también la primera serie de casos de UTx en humanos, llevado a cabo por nuestro grupo, con la consiguiente obtención de recién nacido vivo.

Los principales hallazgos de los estudios arriba mencionados fueron:

-Que el inhibidor de la calcineurina ciclosporina-A, pero no tacrolimus, disminuye las tasas de ovulación y la implantación en modelos experimentales murinos.

-Que en modelos murinos, el desarrollo evolutivo de las crías nacidas de madres trasplantadas de útero (alogénico) es normal desde un punto de vista fenotípico.

-Que en pacientes trasplantadas, el trasplante y la inmunosupresión tienen moderado o ningún efecto sobre la aparición de resultados perinatales adversos cuando se controlan otros factores de confusión vinculados a la condición basal de la madre.

-En esta tesis se ha demostrado por primera vez que el trasplante de útero es una opción viable para el tratamiento de la AUFI, permitiendo obtener nacidos vivos tras trasplante alogénico de útero en humanos.

En resumen, los hallazgos de esta tesis permiten abrir una nueva puerta a la maternidad genética para las mujeres que sufren de este tipo de infertilidad, especialmente en contextos socio-geográficos donde no se permite la subrogación. Igualmente los estudios incluídos en la presente tesis facilitan datos de importante relevancia clínica que pueden ser tenidos en cuenta a la hora de programar el futuro reproductivo de pacientes trasplantadas de órganos sólidos en general.

3 ABBREVIATIONS

- APC: antigen-presenting cells
- ART: assisted reproductive techniques
- ATP: Adenosine triphosphate
- AUFI: absolute uterine factor infertility
- BMI: body mass index (weight in kg/(size in m)^2
- CaN: calcineurin
- CINs: calcineurin inhibitors
- CKD: chronic kidney disease
- CMV: cytomegalovirus
- CyA: cyclosporine-A
- DES: diethylstilbestrol
- DNA: deoxyribonucleic acid
- ESHRE: European Society of Human Reproduction and Endocrinology
- ESRD: end-stage renal disease
- FIGO: International Federation of Obstetricians and Gynaecologists
- Gy: Gray. It is a unit of ionizing radiation dose and it is defined as the absorption of one joule of radiation energy per one kilogram of matter.
- h: hours
- IL: interleukin
- IUA: intrauterine adhesions
- IVF: in vitro fertilization
- LH: luteinizing hormone,
- MHC: histocompatibility complex
- MMF: mycophenolate mofetil
- MRKH: Mayer-Rokitansky-Küster-Hauser
- mTOR: mammalian target of rapamycin

- NODAT: new onset diabetes after transplantation
- PER: Perfadex® (preservation solution)
- pET: personalized embryo transfer
- RIN: ringer acetate
- SIR: standardized incidence ratios
- SET: single ET
- TCR: T cell receptor
- uNK: uterine natural killer cells
- UTx: uterus transplantation
- UW: University of Wisconsin (preservation solution)
- WOI: window of implantation
- WNT: wingless/int

4 INTRODUCTION

Despite major breakthroughs in infertility treatment and assisted reproduction over the last 25 years, absolute uterine factor infertility (AUFI) still lacks reliable treatment. Today, the options for motherhood for uterine factor infertile patients are gestational surrogacy or adoption, the former being the only way to achieve genetic motherhood. Unfortunately for AUFI patients, surrogacy is not considered a legal option in many countries, including Spain and Sweden. In this scenario, AUFI patients can choose to adopt, accept their infertility, or travel to a different country to undergo a procedure considered illegal in their own country. This latter option carries the additional risk that the authorities of the country of residency will deny citizenship to newborns [9].

Advances in the field of transplantation surgery, such as the introduction in the early 1980s of the first calcineurin inhibitor, cyclosporine, as an effective immunosuppressant greatly improved long-term survival for most types of solid organ transplants [10]. The presence of optimized immunosuppressant protocols, which are also able to control rejection of strongly immunogenic tissues [11], has allowed the successful achievement of various types of vascularized composite tissue transplantations of, for example, the face, hand, forearm, abdominal wall, and larynx [12]. This group of transplantations are of course not vital but are rather quality-of-life-enhancing transplantations. It has been proposed that uterus transplantation (UTx) should be incorporated into the group of quality-of-life-enhancing transplantations [3, 7], since the aim of the procedure is to treat permanent infertility, which is associated with impaired quality-of-life [13].

The aim of this dissertation is to describe in detail the basic and clinical research related to fertility after allogeneic uterus transplantation, from experiments carried out in rodents to experience acquired through human models. These experiments include work aimed to characterize the effect of

UTx and immunosuppressive regimens on ovulation, implantation, pregnancy development and offspring phenotype, while models have utilized epidemiological data to evaluate the effect of immunosuppression on the pregnancy outcome. This dissertation also describes the first successful case series, carried out by our group, involving a live birth after UTx in human patients.

4.1 Potential patients. The absolute uterine factor infertility.

AUFI refers to a type of infertility that is 100% attributable to the absence of a normal uterus, either anatomical or functional, which prevents the implantation of an embryo or the ability to carry a term pregnancy. AUFI can be congenital or acquired. There are also some uterine abnormalities, whose presence can cause variable degrees of infertility or subfertility, although it its difficult to prove that such uterine abnormality is the major cause of infertility in each specific case. Patients belonging to the latter group may often benefit from other established medical or surgical treatments, and uterus transplantation should only be considered as the last resort when all other therapeutic options have failed.

Because of the above-mentioned issues, we can describe a cause-specificity when it comes to uterine infertility. The prevalence of uterine infertility among patients of childbearing age is not exactly known but it is likely to be significant, with a recent estimation of 12,000 – 15,000 uterine-infertile patients in the United Kingdom [3]. This estimation would indicate the presence of more than 150,000 uterine infertile patients in Europe, although obviously only a portion of these would have the desire to obtain a pregnancy through uterine transplantation (UTx). As a general rule, the more serious the cause of uterine infertility, the less prevalent the condition (Table 1). Women lacking

an anatomical uterus will naturally belong to the AUFI group. Women with an anatomical uterus are considered to have a relative uterine infertility.

Table 1. Main causes of infertility of uterine origin susceptible of being treated by UTx.

Absolute infertility (only treatable by adoption, surrogacy or UTx)		
Cause	*Prevalence (%)*	Related infertility/sterility (%)
Uterine agenesia	0.0002	100
Leiomyomas requiring hysterectomy	1	100
Post-partum hysterectomy	0.04-1.25	100
Hysterectomy for cervical neoplasia	0.00004-0.0001	100
Uterine hypoplasia	0.038	100*
Relative infertility (Patients in who UTx should only be considered as a last-line treatment)		
Cause	*Prevalence (%)*	Related infertility/sterility (%)
Intrauterine adhesions	<1	70
Unicornuate uterus	0.3-0.5	56.3
Didelphys uterus	0.1-0.3	40
Leiomyomas	21-26	40
Septate uterus	0.8-1.4	38
Bicornuate uterus	*0.7-1.3*	37.5
Arcuate uterus	*1.3-6.2*	17.3

Prevalences estimated with data from references [14-24]

**Probably close to o 100%. Estimation done based on case reports.

4.1.1 Women with no uterus

Hysterectomy during fertile age is the most prevalent cause of uterine infertility. It is the most frequent gynecological surgery with around 600,000 procedures per year performed in the United States of America, with more than 40% of these patients being under the age of 44 [25]. There are numerous causes motivating a hysterectomy at fertile age; these are described below.

4.1.1.1 Uterine neoplasia

Cervical cancer is, from a global perspective, the most common malignancy of the female genital tract. It is caused by a persistent infection of oncogenic HPV

strains [26]. Despite current screening programs, cervical cancer affects a large proportion of women of fertile age. It is reported that more than 30% of cervical cancer patients are diagnosed before 40 years old [20, 27]. The surgical treatment of cervical cancer is limited to the initial stages (up to stage IIA). Patients with squamous cell carcinoma of the cervix with a size < 2 cm and with invasion depth < 10 mm can benefit from fertility-sparing surgeries like the vaginal trachelectomy [28] or abdominal trachelectomy [29], but for patients with more advanced disease, the treatment of their malignancy is radical hysterectomy of the uterus and parametria. The ovaries are spared in almost all cases occurring during fertile age, since the risk of metastatic spread to the ovaries is extremely low for squamous cell carcinoma as well as for adenocarcinoma at these stages.

Other malignancies of the uterus, such as sarcoma or endometrial cancer, are very infrequent during the reproductive years and represent less than 3% of uterine malignancies in women below the age of 40 [30]. Initial stages of endometrial carcinoma of well-differentiated grade can be successfully treated by a fertility-sparing approach with high-dose progestins for 3-6 months, which should be repeatedly monitored with ultrasound, hysteroscopy and endometrial biopsies [31]. Rhabdomyosarcoma of the cervix can also be successfully managed with intensive chemotherapy and tumor excision, without the need for a hysterectomy [32]. Despite these two examples of fertility-sparing approaches, the surgical treatment of endometrial cancer beyond stage I and sarcomas typically includes hysterectomy and, in many cases, oophorectomy.

4.1.1.2 Peripartum hysterectomy

Emergency peripartum hysterectomy is performed to save the life of the mother in situations of severe bleeding due to uterine rupture, atony, invasive malplacentation or uncontrolled bleeding at caesarean section. The incidence of hysterectomy in conjunction with birth (either vaginal delivery or cesarean

delivery) is around 5 in 10,000 deliveries [18]. Cesarean delivery is an independent risk factor for emergency hysterectomy [33]. Although peripartum hysterectomy rates have decreased over the last decade, cesarean delivery rates have increased, and therefore the total number of peripartum hysterectomies remains constant [33]. In a recent population-based study done in the Calgary region of Canada, the hysterectomy rate at childbirth decreased from 1.3% to 0.8% from 1999 to 2006. Nevertheless, during that period there was an increased number of deliveries (from 12,370 to 15,720) and an increasing rate of cesarean section (from 20.7% to 28.4%). The total number of emergency hysterectomies has remained constant (16 hysterectomies performed in 1999 and 13 in 2006), which is probably due to an increased knowledge and use of compression sutures and intrauterine compression balloons to stop bleeding and thereby save the uterus [33].

4.1.1.3 Myoma requiring hysterectomy

Myomas, also known as fibroids, are benign tumors of the muscle layer of the uterus. Histologically, fibroids are formed by smooth-muscle cells that are clonally expanded and surrounded by a disorganized extracellular matrix, which composes the major volume of the myoma and is also responsible for most of the continuous tumor expansion [34]. The myoma is hormonally dependent, developing after puberty and tending to shrink after menopause. The prevalence of uterine myoma increases with age [35] with a frequency of around 8% in women between 33 and 40 years [36]. Risk factors associated with the presence of myoma include age, African American ethnicity, earlier age of menarche, and nulliparity [37]. Myomas are the leading cause of hysterectomy in the United States at a cost of up to $34 billion per year [38]. Approximately 1% of all women between 30 and 34 years, and around 2.5% of those between 35 and 39, are hysterectomized due to fibroids [19]. Symptoms such as excessive menstrual bleeding, anemia, and pelvic pain in women with

large myomas are the main causes for hysterectomy during the premenopausal period. Additionally, myomas [39, 40] are a factor behind infertility (see paragraph “myoma not requiring hysterectomy” below).

4.1.1.4 Mayer-Rokitansky-Küster-Hauser (MRKH) Syndrome

The MRKH syndrome is characterized by the absence of a uterus or the presence of a rudimentary solid bipartite uterus, and is always found in combination with an absence of the upper two-thirds (superior to the hyminal ring) of the vagina. MRKH syndrome accounts for less than 3% of all Müllerian malformations [23] and is present in 1:4500 females [41]. There are three subtypes of MRKH syndrome: a) The typical form, without any extragenital malformation (50% of patients); b) the atypical form, with associated malformations in the renal system (20% of patients), and c) the severe form, with associated renal and skeletal malformations (30% of patients)[42]. Girls with the MRKH syndrome have a normal karyotype and can have normal offspring without urogenital malformations when they make use of surrogacy using their own eggs [43]. Although the occurrence of the MRKH syndrome was thought to be purely sporadic, in familial cases it seems to be inherited as an autosomal dominant trait with incomplete penetrance and variable expressivity. Different genes from the WNT family and deletions and rearrangements in larger DNA regions have been related to this syndrome [44, 45].

Women with the typical MRKH syndrome would be suitable patients for UTx in the future. Provided a neovagina of sufficient length exists, it could easily be surgically connected to the cervix of a transplanted uterus.

4.1.2 Women with anatomical (but not functional) uterus

4.1.2.1 Radiation damage

Radiotherapy, given either as total body irradiation or as local irradiation on the pelvis, causes considerable (around 60%) reduction in uterine volume [46], and

this shrinkage is irreversible [47, 48]. This results in an inability to conceive or in an increased rate of miscarriage and late pregnancy loss [49]. In a series of 15 patients undergoing infradiaphragmatic radiotherapy, Vernaeve et al. found similar implantation rates (31%) when compared to a control group (35.8%) [50]. Noteworthy was the high rate of adverse perinatal outcome (53%), including stillbirth, preterm delivery, preeclampsia and placental abruption. In addition to the above-mentioned effects of radiation on the uterus, it should also be noted that radiation doses as low as 5 Gy on the ovaries compromise gonadal function in most women [51]. Therefore, genetic maternity in this group of patients could only be achieved if a fertility preservation technique has been carried out before radiotherapy [52]. In the case of a radiation-damaged uterus in a prospective UTx patient, the organ could be replaced in a combined procedure of hysterectomy and transplantation. During hysterectomy of a radiation-injured uterus, the long proximal ends of the uterine arteries and veins could be spared to facilitate the transplantation surgery. A concern with this type of patient, however, are the surgical difficulties related to scarring, adhesions and impaired healing induced by radiation [53].

4.1.2.2 Myoma not requiring hysterectomy

The prevalence of myomas in infertile women is much higher than the previously mentioned 8% of the general population of women aged 33 to 40 years. In a study including patients undergoing IVF, the incidence of myomas during a one year observation window was 26.7% [54].

In general, it is accepted that a subserosal leiomyoma does not affect gestation, while submucous or intramural leiomyomas, the latter when protruding into the endometrial cavity, are associated with decreased rates of implantation and pregnancy [34]. When a myoma is intramural and does not distort the endometrial cavity, there seems to be a mild negative effect on fertility. This was observed in a recent meta-analysis which showed a 21% relative reduction

in live birth rate in women with non-cavity-distorting intramural fibroids compared with women without fibroids (RR: 0.79, 95% CI: 0.70– 0.88), and this effect remained even when only high quality trials were included in the analysis (RR: 0.60, 95% CI: 0.41–0.87) [55].

With respect to surgical myomectomy of large/multiple intramural myomas, a prospective but non-randomized study showed fertility in around 60% of the operated patients [56]. Thus, our current practice is to perform a laparoscopy in patients with fibroids classified by the International Federation of Obstetricians and Gynaecologists (FIGO) as stages 3 to 6 if they have a size (≥4 cm) that can easily be identified and removed by laparoscopy (Munro et al., 2011). Myomectomy surgery might also be considered for smaller fibroids, especially if there are previous failed assisted reproductive techniques (ART) attempts, but only if surgery can be performed with a low probability of complication. If the endometrial cavity is not reached during a myomectomy surgery, pregnancy can be attempted 3 months after surgery. However, if the endometrium is visualized during the surgical procedure, it is generally recommended that 6 months are allowed for uterine healing prior to pregnancy attempts [34].

Patients that remain infertile despite myomectomy, including those who have undergone hysterectomy [19, 24] because of large symptomatic myomas (described in the paragraph “myomas requiring hysterectomy”), belong to the group of myoma-related uterine infertile patients that could be treated by UTx.

4.1.2.3 Asherman’s syndrome

Asherman’s syndrome, also known as intrauterine adhesions (IUA), is characterized by the presence of intrauterine adhesions in the endometrial cavity as a result of a previous damage caused by a variety of factors. The prevalence of the syndrome is around 1.5% among fertile-aged females [17], leads to a miscarriage rate around 40%, and causes infertility in around 50% of women [57]. Asherman’s syndrome is usually secondary to endometritis [58],

surgical curettage during legal abortion or postpartum [59], or surgical hysteroscopic procedures [60]. The treatment of choice for intrauterine adhesions is hysteroscopic adhesiolysis which can effectively cure infertility in mild, moderate and severe IUA with approximate rates of 90%, 70% and 30%, respectively [16]. From these data it can be estimated that around one-third of the total population with IUA have irreversible uterine infertility, and these women may be treated by a combined hysterectomy-UTx procedure.

4.1.2.4 Congenital uterine malformations and uterine infertility

Congenital uterine malformations occur because of disturbances during fetal life in the formation, development or fusion of the Müllerian (paramesonephric) ducts. The Müllerian duct malformations may cause infertility and also increase the risk of adverse obstetric and perinatal outcomes [61]. It is estimated that the prevalence of uterine malformations in the general population is around 5-6.7%, a number which is not so different from that of the infertile population (7.3%) [22]. This implies that a majority of these malformations do not have any negative impact on fertility, with the most obvious example being the partial septum of the uterus, which is surgically correctable as discussed below.

On the other hand, less prevalent but more severe forms of uterine malformations such as the MRKH syndrome, the hypoplastic uterus, the unicornuate uterus, the uterus with a complete septum, and the bicornuate uterus may have a considerable negative effect on fertility. The prevalence of the above-mentioned conditions increases to 16.7% in women with recurrent miscarriage [22]. Sorted by prevalence, the most frequent forms of uterine malformations are:

-Septate uterus: This is the most prevalent type of structural congenital uterine anomaly among infertile women [22]. It accounts for more than 30% of all uterine malformations and is the result of incomplete resorption of the central parts of the two fused Müllerian ducts. Spontaneous abortion occurs in about

80% of pregnancies in untreated septate uteri [62]. However, hysteroscopic resection is an effective treatment of the sepatate uterus which substantially decreases the miscarriage rate [62].

-Bicornuate uterus: This is caused by the absence of fusion of the two Müllerian ducts. It accounts for 25% of all uterine malformations [23]. The rate of spontaneous abortion among women with bicornuate uteri is around 35% [63]. Abdominal metroplasty can result in an 82% live birth rate in women with previous miscarriages [21], although this surgery implies a higher risk of uterine rupture during pregnancy. There are also series in the literature in which similar reproductive outcomes have been described in women with bicornuate uterus treated with metroplasty compared to those without surgical intervention [64].

-Unicornuate and uterus didelphys: Together these comprise around 20% of uterine malformations [23]. Disturbed development of one of the Müllerian ducts can result in the unicornuate uterus, with or without a contralateral rudimentary uterine horn. A total failure of fusion of the Müllerian ducts results in uterus didelphys, that is, two separate uterine horns without a common cavity. The common feature of these two entities is that a given uterine cavity (one in the case of the unicornutate uterus, and two in the case of a didelphys uterus) usually has a smaller size when compared to a normal uterus, with an increased miscarriage rate of 30% and decreased live birth rate of 50% being reported [23]. Surgery does not seem to improve the pregnancy potential of the unicornuate/dideplhys uterus [65]. Thus, a considerable proportion of these patients are unable to carry a pregnancy into the third trimester.

-Other: T-shaped uterus and hypoplastic uterus are two infrequent forms of uterine malformation. T-shaped uterus is mainly caused by exposure to diethylstilbestrol (DES) during the fetal life, while hypoplastic uterus is usually associated with abnormal karyotypes (e.g. Turner syndrome) or genetic abnormalities like the Swyer syndrome [34]. The mechanism(s) underlying the

reduced reproductive performance in women with hypoplastic and T-shaped uteri might be a reduced uterine blood flow supply, the association with other malformations, and/or the absence of a functional myometrial layer [34]. Live birth rates ranging from 2 to 21% have been described in this group of patients.
The arcuate uterus is not included in this review since it could be considered a subtle abnormality or a normal variant with minimal or no clinical significance [15, 66].
As pointed out in the previous paragraphs discussing malformation-specific pregnancy rates, a considerable portion of patients will remain infertile even with corrective surgery, and it is these patients who could benefit from UTx as a solution to achieve genetic motherhood.

4.1.2.5 Functional dysregulation of the uterus

Endometrial receptivity is a transient status of the luminal epithelium that renders the endometrium suitable for blastocyst attachment, initiating the implantation process [34]. The human endometrium is receptive to embryo implantation during a narrow time frame of the menstrual cycle referred to as the window of implantation (WOI). The WOI in humans usually occurs 3 to 7 days after progesterone rise, which corresponds to 5 to 7 days after LH surge [67].
It is known that the WOI can be displaced, causing a mismatch between the embryo developmental stage and endometrial receptivity, and thus leading to infertility. In the field of IVF, embryo transfer that takes place outside the WOI (during pre-receptivity or post-receptivity status), is not conducive to implantation and further pregnancy. Nowadays, diagnostic tools provide a genetic expression profile of the endometrium, and personalized embryo transfer (pET) in a given patient is now possible. Nevertheless, despite the use of these tools, 25% of patients remain infertile [68].

4.2 Historical perspective of uterus transplantation

4.2.1 Previous attempts at uterus transplantation in the human

The only two previous attempts of UTx carried out in the human prior to the clinical trial done by our group were conducted in 2000 and 2011. Both cases are described in detail in the following paragraphs.

4.2.1.1 First attempt

The first human UTx attempt was undertaken in Saudi Arabia in the year 2000. The uterus of a 46-year-old living donor was transplanted into a 26-year-old woman, who at the age of 20 had undergone a peripartum hysterectomy [69]. The surgery of the donor should be regarded as partly successful since the uterus with vascular pedicles could be harvested, although they were of insufficient lengths for direct anastomosis to the external iliacs. Although a ureteric injury occurred, it was repaired during the procurement surgery. The uterus survived for 3 months but was then removed because of necrosis [69].

No exact details were given concerning the hysterectomy procedure at organ procurement, although they naturally had to involve bilateral ureteric dissections to release them from the uterine cervix and from the attachments to the uterine arteries and veins. However, during uterus retrieval surgery, only short pedicles of the uterine arteries and veins were obtained, which required these vessels to be lengthened during back-table preparation by end-to-end anastomosis of vascular segments (~5 cm length) of the saphenous veins. From the report is seems that the uterine veins and uterine arteries were elongated with saphenous grafts on each side. It was not mentioned whether this corresponded to venous connections to the external iliac veins on each side, or if the saphenous extensions of the uterine veins were joined on the back table to create only one venous outlet on each side. This latter procedure would naturally make the anastomosis surgery faster, thereby decreasing the harmful

warm ischemic period. The transplantation procedure was accomplished by end-to-side anastomosis of the vessels of the graft to the external iliac vessels on both sides. The team that performed this human UTx did not have any previous publication record in the UTx field, although they stated in the paper that they had practiced the procedure by performing uterus auto-transplantation in both goats and baboons previous to the trial [69].

This transplantation case presented several pitfalls of paramount importance:

1) The surgical technique was not properly optimized:

-As mentioned above, eight anastomoses may have been used during surgery [69], with each being a potential site for thrombosis. Moreover, the greater number of anastomoses, the longer the warm ischemic times, resulting in diminished graft survival and functionality [70]. After proper research in different animal models, including non-human primates [71] and preclinical human studies [72], we learned that UTx can be achieved with as little as 2 (aorta and vena cava) or 4 anastomoses (2 uterine veins and 2 uterine arteries without the need for any extra vascular segment).

-The Fallopian tubes were kept in the graft [69]: We have learned from non-human primate experiments [73] that UTx surgery affects the functionality of the Fallopian tubes with no patency observed after a six-month follow-up. A suboptimal oviductal function may increase the risk of ectopic pregnancy, and if this occurs in a graft it will be a very difficult surgery to perform, since it is unlikely that laparoscopy can be used, and a laparotomic procedure will involve extensive surgery to dissect adhesions before reaching the oviduct of the ectopic pregnancy.

-During the recipient surgery, the cervix was sutured first [69]. This surgical gesture limits the graft mobility from the outset and is likely to extend the surgical time.

2) The follow-up of the transplanted patient lacked sensitivity to detect rejection: Fageeh et al. used the CD4/CD8-T-cell ratios in peripheral blood and

blood flow by Doppler ultrasound to assess graft rejection [69]. It is quite likely that the CD4/CD8 ratio has a low sensitivity for detecting rejection, as does a uterine allograft, as previously shown for other transplants [74]. Moreover, Doppler measurement of uterine blood flow has not been evaluated as a marker of rejection in any animal model.

It is difficult to precisely understand the cause of uterine demise in this human case [69, 75]. Thrombosis of the transplanted graft with secondary necrosis is a common finding after rejection [76], and once necrosis has occurred it is difficult to distinguish the cause. Vascular graft thrombosis is known to be one of the earliest events after allotransplantation and rejection [75, 77]. Specific anti-endothelial antibodies may cause endothelial disruption and thrombosis even in the absence of anti-HLA or anti-ABO antibodies [78]. Thrombosis may also be due to suboptimal vascular connections, with the saphenous grafts being critical sites in this specific case. In the report [69], the authors suggested that uterine necrosis was not associated with rejection, and instead proposed that prolapse of the organ led to kinking of the vessels and secondary thrombosis.

4.2.1.2 Second attempt:

In 2011, the world's second human UTx was attempted. The team performing the procedure was led by a plastic surgeon with previous experience in composite tissue allograft transplantation [79]. Although they did not have any previous experience in animal models, they claimed to have used the preclinical experience reported by our group [80]. In Antalya, Turkey, a 21-year old MRKH-patient received a uterus from a 22-year-old deceased donor [80]. The recovery from the multi-organ donor lasted 2 hours and the uterus was the first organ to be recovered. The fact that the uterus was the prioritized organ is not without controversy, as this would lead to the possible decreased quality of other vital organs to be harvested, such as the heart, lungs and liver. The

transplantation procedure took 6 hours and included bilateral end-to-side anastomosis of the common iliac vessels of the graft to the external iliac vessels. Immunosuppression was thymoglobulin for 10 days and then triple-maintenance therapy by prednisolone, mycophenolate mofetil and tacrolimus. Eighteen months after UTx, embryo transfer attempts were initiated. The patient has had multiple IVF attempts, but with only two very early miscarriages as the end result [81]. The reason for the pregnancy failure in this case is unknown, but an important factor to take into consideration is that a nulliparous uterus was transplanted, and the capacity to carry a normal pregnancy of this uterus has never been demonstrated. This means that there may be some uterine-specific factor of this nulliparous woman that caused pregnancy failure, which could be structural or biochemical.

4.2.2 The IDEAL concept

The first two UTx experiences raised an ethical debate [82, 83], and also boosted the research activities within the field of UTx [7]. The Saudi Arabian case and the debate surrounding it, both in the scientific community and also in media, was a clear signal to FIGO to provide some ethical guidelines for the development and clinical introduction of Utx [84]. These guidelines highlighted safety issues before a new clinical attempt of UTx was carried out in humans, with explicit mention of the fact that UTx should only occur after significant and adequate research in appropriate large animal models, including primates [84]. At that stage, our group had already published more than 16 research articles of experiments done in animal models evaluating different aspects of UTx [7]. The guiding philosophy of our group has always been that of minimizing any risk prior to clinical application. This philosophy is also reflected by the IDEAL framework [8, 85, 86], in which surgical innovations follow an escalating step-by-step evaluation approach, just like when testing

and introducing new pharmaceuticals, before they become a clinical reality. The IDEAL acronym stands for:

-Innovation: This is when a surgical technique is described for the first time. This point includes the proof of concept in the form of case-reports, or in the form of intensive pre-clinical research when the risks are unacceptable.

-Development: At this stage, clarification of inclusion an exclusion criteria, modifications of the original technique and report of relevant outcomes must be done.

-Exploration: This stage occurs after the first case series. The main outcomes of the procedure are usually known, but experience in larger groups of patients (usually up to some hundreds) is needed. This is the stage when the procedures start to be performed in different centers and learning curves are evaluated. Prospective uncontrolled trials should start at this stage.

-Assessment: At this stage, the new surgical technique should be fully developed and can be compared to other treatment alternatives. Randomized controlled trials are the default type of study to achieve this goal. Nevertheless, these kinds of studies are difficult, unethical or sometimes unnecessary simply to demonstrate the superiority of one intervention over the alternative.

-Long-term study: This stage is devoted to the detection of long-term or infrequent adverse effects of the surgical technique. It usually involves the creation of patient registries, and it allows for risk adjustment for patients' comorbidity. It also provides a surgeon or surgical unit a means for tracing/evaluating their own performance over time.

The IDEAL guidelines strongly encourage "the use of experimental rather than observational designs" [8], and this is how our UTx program was based. The first years involved the development of different animal models including rodents, different domestic species, non-human primates and human preclinical models with the use of non-rejection transplantation models to study aspects of surgery, ischemia-reperfusion and pregnancy after syngeneic UTx [70, 87-96].

We also developed animal models of allogeneic UTx to characterize rejection processes and immunosuppression protocols, and finally we conducted the studies that make up the core of this thesis: Animal models designed to achieve the proof of concept of live offspring after allogeneic uterus transplantation under immunosuppression [71, 97-102]. All of the preclinical studies performed before the experiments included in this thesis are summarized in the following paragraphs of the introduction.

4.2.3 Surgical techniques and surgical models

4.2.3.1 Early studies on uterus transplantation

In the 1960s and 70s, intensive research was dedicated to tubal factor infertility prior to the development of IVF. This research focused on reconstructive surgery [103-106], but also investigated methods to replace the affected Fallopian tube with a functional one using oviductal transplantation [107-115]. This was the origin of the modern research on UTx. At that time, however, our knowledge of rejection mechanisms and the immunology of transplantation was far inferior to what we know today. These pioneering studies were therefore important contributors to our understanding of the surgical technique, the selection of the vessels to anastomose, and the assessment of the different methods of performing anastomosis. A summary of these experiments and the different animal models used follow below.

-Dog: The first procedure for surgical isolation of the uterus (with oviducts and ovaries) followed by reanastomosis was described in the dog [116, 117]. The blood vessels from the uterus were isolated up to and including the common internal iliac arteries and veins. These experiments were not technically proper transplantation experiments since the uterus was connected to the vagina throughout the procedure. Nevertheless, they contributed to the development and description of the dissection procedure. Later on, utero-tubo-ovarian grafts

in the dog were separated from the other vessels of the pelvis [118, 119], but were still flushed in situ under warm conditions. Thus, the organ was never removed from the abdominal cavity of the animal in these auto-transplantation experiments. Reanastomosis was performed end-to-end (ends of the common branch of the internal iliac artery) and end-to-side (bilateral internal iliac veins to common iliac vein) using 5-0 silk sutures. In these dog models of uterine vascular re-anastomosis, a cumulative pregnancy rate of 11% was seen in the studies aimed at pregnancy outcome [116, 117, 119]. Subsequent experiments described how to include lower portions of the aorta/cava [120, 121] or common iliac vessels [118, 122] in the vascular pedicle of the graft.

A small number of studies in the dog examined omentopexy [108, 123], rather than of vascular anastomosis, as a method for vascularization of the uterus. The survival of auto-transplanted uteri was much lower after omentopexy than vascular anastomosis [119] indicating that the uterus is dependent on immediate blood flow and cannot survive in warm ischemic conditions for the few days it takes for neovascularization to connect to the vascular bed of the uterus.

-Rabbit: The experiments carried out in the rabbit model [124] are of limited value due to the fact that they were avascular, with transplantation of the uterus into the broad ligament, and also by the fact that many of the transplantations were done in an allogeneic setting. Thus, the results in terms of graft survival were very poor and therefore offered little guidance for future research.

-Sheep: In this era of preliminary UTx experiments the sheep model was not used for uterine transplants. Nevertheless, there were some experiments describing vascular anastomosis of the utero-tubal vessels to the carotid artery and jugular vein when the uterus with adnexae was transplanted to this heterotopic position [125, 126]. The model allowed easy access to the venous effluents and was primarily used to study the uterine regulation of corpus luteum function. We later adapted part of this technique in our experiments of UTx in the sheep model, as discussed further below.

-Non-human primates: It is difficult to achieve accurate conclusions from the single non-human primate UTx study done in the 1970s. In this study, auto and allotransplantations of the uterus were performed in rhesus macaques [127]. The limitation of this study was that avascular transplantation was performed, with the uterus wrapped inside the omentum, rather than performing proper vascular anastomoses. Surprisingly, in the auto-transplanted animals the uteri maintained normal size and menstruation occurred. However, no pregnancy occurred despite breeding attempts for 10 months [127]. It was suggested that the reason for the negative pregnancy results was tubal blockage, caused by ischemic injuries during the period between organ procurement and reimplantation. In the subset of allogeneic transplanted monkeys that did not receive any immunosuppressive therapy, major rejection signs were present throughout the uterus and in oviducts, and at later stages full necrosis was observed [127].

4.2.3.2 Modern studies on uterus transplantation

The main feature of modern research in UTx (after 1985) is that the different potentially harmful events that may lead to unsuccessful transplantation have been analyzed separately. This allows conclusions to be drawn concerning each individual step of the procedure. These potentially damaging events include surgery at organ recover, ischemia-reperfusion damage, surgery at transplantation, and of course rejection and possible effects of immunosuppressive medication. During this modern era of UTx research, well-designed experiments have used syngeneic and auto-transplantation models to optimize the UTx procedure from a surgical standpoint and to characterize ischemic preservation. The data acquired from the above optimally conditioned animal models was then used as an experimental control situation, where effects of rejection and immunosuppression were added to the allogeneic transplantation model in order to characterize the these effects in UTx, separate

from the major surgical and ischemic events. More recent research in the UTx field has been conducted in several animal models including rodents (mouse, rat) [89, 90, 92, 98], large domestic species (sheep, pig) [94, 95, 128, 129], non-human primates (baboon, macaque) [71, 88, 130, 131], and even in humans [72, 83, 96, 132].

The surgical experience acquired during this period is reviewed in the following pages, with specific focus on syngeneic and auto-transplantation models. Experience concerning ischemia-reperfusion damage and rejection/immunosuppression will be reviewed in the following sections: "Rejection in uterus transplantation", "Resistance to cold ischemia in uterus transplantation", "Resistance to warm ischemia in uterus transplantation" and "Immunosuppression".

-Mouse: In our group, we initially chose the mouse as a suitable species to perform basic UTx research because of the availability of gene-modified strains, easy embryo-transfer, and the accessibility of specific recombinant proteins and monoclonal antibodies. The retrieval of the uterine graft, with one horn excised, included unilateral dissection of a vascular pedicle with the uterine vessels and more proximal vessels, all the way up to the mid-abdominal section of the aorta and the vena cava [90]. The duration of the uterus retrieval procedure decreased from 60 to 45 minutes in the primary experimental series. The uterus was kept cold during back-table preparation and during surgical preparation of the recipient, with a cold ischemic time of about 35 min. The second warm ischemia, during vascular anastomosis, lasted for about 50 minutes.

The uterus with vascular pedicles, including vessels from the uterus, and up to and incorporating the subrenal parts of the aorta and vena cava (diameters of around 0.7 mm and 1.5 mm, respectively), were attached end-to-side (11-0 nylon sutures) to the aorta/cava of the recipient mouse. In the last series, almost 90% of the grafts of the surviving animals showed successful transplants [89,

91]. In all the experiments the native uterus was kept and served as an internal control. The grafted uterus was placed in a heterotopic position, somewhat higher up in the abdomen. The initial study kept the cervix inside the abdomen to avoid infections ascending through an exteriorized cervix [90]. However, the drainage of cervical/uterine cavity fluid was not sufficient in this model and in many cases the uterus would become swollen by accumulated fluid. Consequently, the surgical method was modified to create at cervical cutaneous stoma through the lower abdominal wall [89, 91], which allowed proper drainage of uterine/cervical fluid and, importantly, without any ascending infections despite the cervical exteriorization.

-Rat: In the original rat models of uterus transplantation, the uterus was isolated en bloc with the ovaries plus oviducts [133, 134] and with vascular trees up to and including aortic and caval stumps. The specimens were flushed in situ with 30 ml of cold Ringer solution and the cold ischemic time was around 30 minutes. The duration of the second warm ischemia period during attachment of the graft is not stated, but can be assumed to be about 60 minutes since the total duration for surgeries was approximately 150 minutes [134]. The uterus was placed orthotopically with vaginal anastomosis followed by vascular connections end-to-side to the aorta and vena cava of the donor with 9-0 sutures [134]. In syngeneic transplants [134], all grafts showed revascularization lasting at least 3 months.

In our original rat uterus transplantation model, a graft consisting of the right uterine horn, the common uterine cavity, and the cervix was transplanted between inbred Lewis rats. This was done by anastomosing the right common iliac artery and vein end-to-side to the mid-abdominal part of the aorta and the vena cava of the recipient by continuous 10-0 nylon suture [133]. The native uterus was left in situ as an internal control and the transplanted uterus was placed in a heterotopic position with the vaginal rim of the graft being connected to a cutaneous stoma. There was an approximate 30% loss of

transplants among the surviving animals (above 95% in the last series of experiments), and the cause of graft loss was exclusively due to thrombosis formation. The duration of uterus retrieval in the rat was around 60 minutes after the initial learning period [133]. The cold ischemia, during preparation of the recipient was about 60 minutes. The second warm ischemia period, during surgery for vascular anastomosis, was around 90 minutes. In these syngeneic transplantation experiments the graft survival was 80% [133].

-Pig: The pig is physiologically similar to humans and is commonly used for surgical training in preparation for human procedures. Our group [94] and others [3] have used the pig in experiments involving a supracervical hysterectomy and dissections of the uterine vessels to a level above the ureters. In our experiments [94], the uterus was flushed with ice cold Ringer Acetate for a total time of 1-2 hours. Anastomoses of the uterine vessels were performed end-to-end using 6-0, 7-0 or 9-0 interrupted sutures [3, 94]. The uterus was fixed to the cervix by interrupted 1-0 polydioxanone [3] or 3-0 polyglactin [94] sutures . Less than 20% of the uteri were judged as being well reperfused [94]. In the properly reperfused uteri, blood gasses and lactate concentrations in uterine blood effluent normalized after 60 minutes. In evaluation of long term function, a gradual and progressive thrombosis developed in the uterine vessels at the anastomosis sites [3].

The dissection of the uterine arteries and their proximal vessels can be achieved up to the level of the aorta when performed in pig cadavers [135]. This approach, in where the internal pelvic vasculature is dissected bilaterally from the uterus to the aorta, was used in an allogenic heterotopic model in mini-pigs [128]. If a deceased donor was going to be used for UTx, this approach would allow obtaining larger vascular patches, permitting easier anastomoses and possibly better inflow/outflow to/from the graft due to the larger diameter of the vessels. Extended information on the allogeneic mini-pig model can be seen in

the paragraph “Characterizing rejection in uterus transplantation-Studies in the era of calcineurin inhibitors” further below.

-Sheep: The uterus of the sheep has one common cavity. This cavity communicates with the vagina through the cervix at its proximal portion, and it divides into two different horns and oviducts at its distal edge, with the fimbriae of the oviducts attached in close proximity to the ovaries. In our experiments [87, 93], we surgically excised the right uterine horn and the vasculature was only dissected unilaterally. This step aimed to decrease the warm ischemic time during surgery by only necessitating unilateral anastomosis of the graft pedicle instead of bilateral anastomosis. The internal iliac artery was dissected free from its proximal end, just distally to the branching of the anterior and posterior divisions. Dissection of the common utero-ovarian vein was made from the uterus to the internal iliac vein. The left oviduct and the ovary were kept in the specimen to allow for long term fertility experiments [95], although this step could have been obviated. The ovarian artery was dissected up to its origin in the aorta, with acquisition of an ellipsoid-shaped aortic patch (size 1x0.5 cm) including the origin of the ovarian artery. The duration of surgery for the procurement was about 3 hours for uterus retrieval and about 4 hours when the ovary was also harvested. Flushing with the preservation solution Perfadex was performed in situ and the graft was cold stored (70 min) before auto-transplantation. The second warm ischemia period, during vascular anastomosis, was about 60 minutes. The arterial end of the graft was the anterior division of the internal iliac vessel (4 mm diameter), which was anastomosed end-to-side to the external iliac artery with a continuous 6-0 polypropylene suture. The larger utero-ovarian vein (8 mm diameter) was then anastomosed end-to-side to the external iliac vein [87, 93]. Outcomes related to ischemia-reperfusion events and fertility are dicussed in “Resistance to cold ischemia in uterus transplantation” and “Resistance to warm ischemia in uterus transplantation”.

Transplantation of the two horns with bilateral anastomosis to the external iliac vessels has also been done in the allogeneic setting of a sheep UTx model [136]. The surgical technique was different, with a hysterectomy being performed with transection of the uterine vessels at a level above the ureters. At transplantation, the uterine vessels were joined end-to-end both on the uterine arteries and veins [136]. If performed in a human, this end-to-end uterine vessel anastomosis technique would only be applicable when a hysterectomy is performed in the same surgical session as the transplantation. Examples of this would be absolute uterine factor infertility due to severe and untreatable intrauterine adhesions or a severely malformed uterus, which cannot carry a pregnancy. The results of this model are commented below in the paragraph "Characterizing rejection in uterus transplantation-Studies in the era of calcineurin inhibitors".

-Non-human primates: During the preparation of this dissertation, our group carried out different experiments in baboons in order to prepare for the human uterus transplantation trial. Although it is not the specific subject of this PhD thesis, a brief summary of this experience will be discussed, as it contributes to a better understanding of the surgical technique presented in papers 7, 8 and 9.

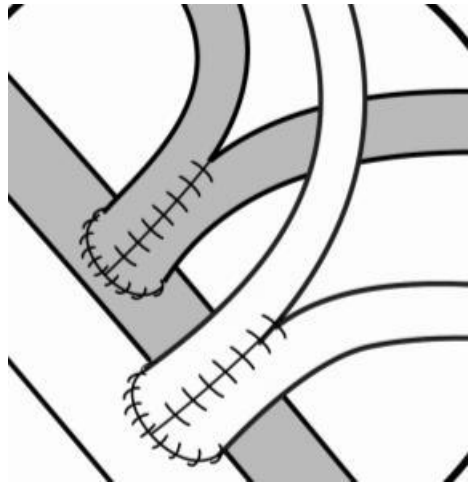


Figure 1. Detail of the principle of anastomosing the ends of the ovarian veins and the anterior branches of the internal iliac arteries side-to-side to create larger vessel ends for anastomosis to the external iliac vessels.

The first series of auto-transplantations included 10 baboons. The surgical technique was similar to that used to perform a radical hysterectomy [88]:

The uterus, Fallopian tubes and ovaries were surgically removed and auto-transplanted into an orthotopic position with vascular connections between the anterior portion of the internal iliac artery, and the ovarian veins to the external iliac vessels on one side [88]. We bisected and sutured (8-0 suture) the ends of the two anterior portions of the internal iliac arteries to create a larger vessel, and a similar procedure was used on the two largest ovarian veins with 9-0 sutures. These two new vessels could then be attached end-to-side to the external iliac vessels with 7-0 and 8-0 sutures using a similar procedure as that routinely used in renal transplantation (Figure 1). This technique initially rendered satisfactory reperfusion of the uterus but with only 2 out of 10 animals showing resumed menstruation. In the rest of the animals the uterus had either shrunk in size considerably, or completely disappeared leaving only a fibrotic scar above the vagina. This was interpreted to indicate that in these 8 animals the blood flow had either been insufficient from the start or that thrombosis had gradually developed.

In a follow up study [73], the autologous transplantation technique of the baboon was modified with extensive dissections of the ovarian veins. This was done to include their inlets into the vena cava and the left kidney vein in order to accomplish a vascular anastomosis with thicker venous walls. Furthermore, the arterial anastomosis at transplantation was modified so as to be unilaterally end-to-end to the internal iliac artery, and the uterus was flushed with a proper preservation solution under cold conditions, rather than the physiological saline previously used. The most important modification was probably that the anastomosis surgery was now performed by a transplant surgeon. Overall, 60% of animals resumed menstruation [73]. These baboons were subjected to repeat mating but pregnancies did not occur, which was most likely due to blocked oviducts.

Kisu's group, working with UTx in Japan, used another non-human primate model of UTx: The cynomolgus macaque [130]. They performed autologous

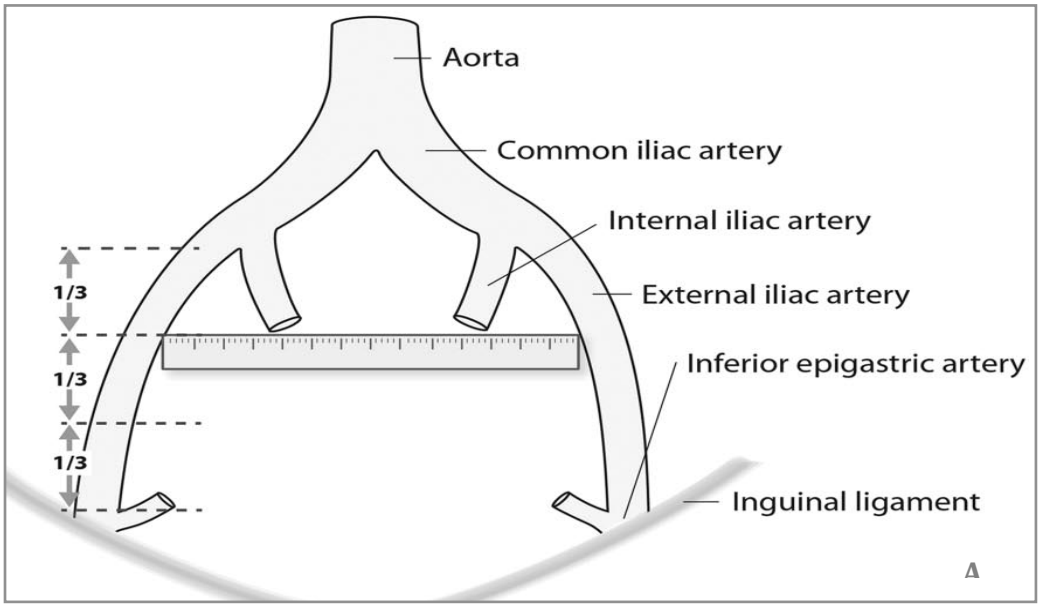
UTx with bilateral anastomosis of the uterine artery and the deep uterine vein to the external iliacs [130]. The study only included two animals and, despite a lengthy operation extending over 13 hours, menstruation resumed in the surviving animal. In a follow-up study, it was indicated that the complete macaque uterus can be adequately perfused with only unilateral anastomosis of one uterine artery and one uterine vein, provided that the side of the dominant blood flow is chosen [131].

-Human: Experiments in the human have also been conducted to develop techniques for harvesting the uterus from live and deceased donors.

In one study investigating the practicability of uterus recovery from deceased donors [83], the complete and bilateral internal iliac arteries and veins were recovered with 2 out of 7 grafts, and the vascular pedicle included the vessels up to the anterior portions of the iliacs in five grafts, but with unilateral loss of uterine vessels in twoof these. The uterine recovery was performed by gynecologists. In our collaborative group, transplant surgeons have recovered uteri from seven multiorgan donors, and in these trials they also recovered vascular pedicles including the complete uterine vessels, internal iliac vessels, common iliac vessels and lower part of the aorta and vena cava (*Tzakis et al., our unpublished data*).

In another study evaluating the feasibility of uterus harvesting during multiorgan donation [132], seven uteri were retrieved after asking for permission in 14 female multiorgan donors. Uteri and the rest of the organs were perfused in situ through the femoral artery, and the uterus was retrieved after other organs. The vesicocervical and recto-vaginal spaces were dissected well below the vaginal fornix, but the graft vascular pedicles were not dissected until the graft was on the back-table [132]. The internal iliac vessels could be preserved in all cases, with the exception of the first retrieval where one vein was lost during organ procurement.

We have also recently conducted a study with vascular dissection of the uterine arteries and veins at open radical hysterectomy in patients with cervical cancer [72]. The study was performed to gain information about whether the uterus could be recovered at live donation with long enough vascular pedicles to eliminate the need for elongations with saphenous grafts as done in the first human UTx attempt [69]. The free lengths of the uterine arteries were almost 70 mm, and those of the uterine veins were 50 mm or slightly longer. Considering that the width of the uterine cervix is about 3-4 cm, these lengths would be sufficient for direct bilateral anastomosis to the external iliacs, with an estimated distance between the vessels of around 100mm (Figure 2). In the event of a postmenopausal live uterus donor, it would also be possible to use one or two of the ovarian veins but obviously oophorectomy would have to be part of the procedure in such a case.



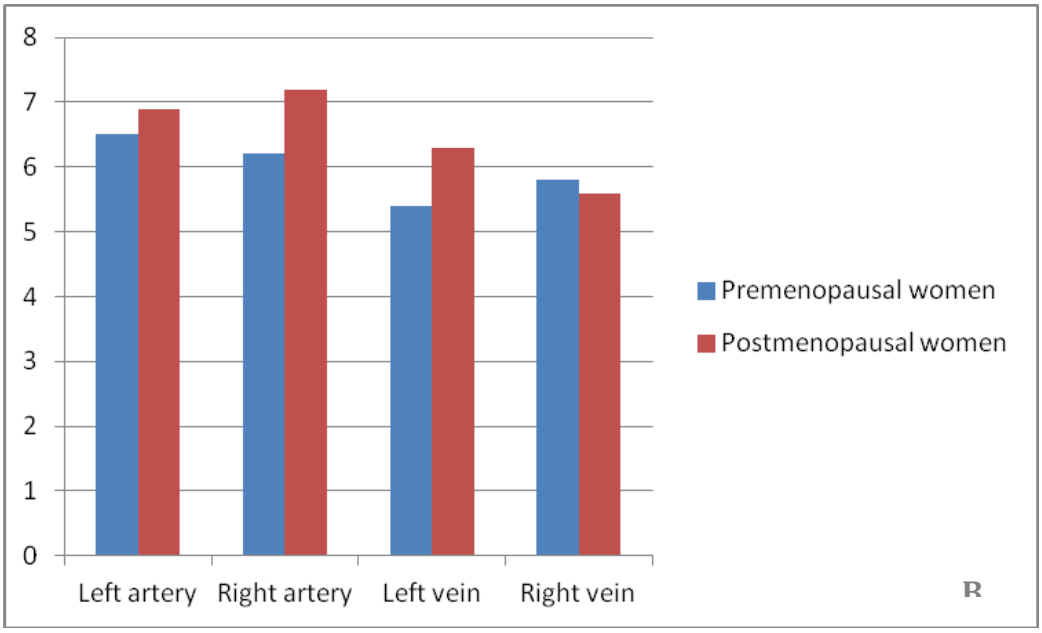


Figure 2A) Ilustration of the inter-external iliac distance, at one third the distance from the bifurcation of the common iliac artery to the inguinal ligament, is indicated. This would correspond to a distance between proposed bilateral anastomosis sites.
Figure 2B) Median lengths of the uterine vessels after hysterectomy for uterine neoplasia. The length of arteries and veins were similar when compared patients according to their menopausal status.

4.2.4 Rejection in uterus transplantation

4.2.4.1 The uterus is not immune to rejection in the allogeneic setting

The uterus is an organ that varies in its population and activity of immune cells during the ovarian cycle [137] and pregnancy [138]. Uterine resident cells of the mother's immune system tolerate a fetus that only shares half of their histocompatibility alleles. The mechanisms behind this tolerance of the uterus of the gestational products (fetus and placenta) are not fully understood. Regulatory T-cells (Druckmann and Druckmann, 2005) and uterine natural killer cells [139] seem to play central roles in maintaining such immunoprivileged status.

Some authors have speculated that since the uterus somehow modulates the maternal immune response during pregnancy, it could also protect itself from immune rejection in a setting of allotransplantation [127]. In the 1970s, using the non-human primate as a model, Scott et al. performed allogeneic transplantation of subtotal hysterectomy specimens with vascularization obtained by omental wrapping and no immunosuppression. After 14 days, major rejection signs were present throughout the grafts [127].

Also in the 1970s, different experiments of allogeneic UTx with the dog as a model were carried out. Yonemoto and coworkers proved that pregnant uteri were equally rejected when compared to non-pregnant uteri in an allogeneic setup [122]. The introduction of immunosuppressants (azathioprine and corticoids) that were used during that era of transplant surgery were not able to prevent uterine rejection, and rejection signs of variable degrees were seen when the grafts were examined at two [120], four [120] or seven weeks [122] after transplantation. The initial rejection signs were perivascular accumulation of inflammatory cells, followed by later loss of the endometrial lining [122]. In late stages, hemorrhage, patchy necrosis, and intravascular thrombosis were present [120, 127].

Already in the 1980s, a comparative study on rejection of the uterus was published with the rabbit as the experimental animal [124]. Does were allocated to receive cyclosporine-A or no immunosuppression. In the control group, 100% of the animals presented rejection signs compared to only 50% of the cyclosporine-treated animals [124].

4.2.4.2 Studies in the era of calcineurin inhibitors

There exist two studies using a combination of cyclosporine-A (CyA) and tacrolimus after allogeneic UTx in the sheep. In one of the studies, ten sheep were treated orally with cyclosporine-A and prednisone starting two days before surgery [136] and discontinued after 2 weeks. Examination of the animals by laparatomy six months after surgery showed pronounced abdominal adhesions in seven out of ten animals. In six out of the ten animals the uterus could be identified, and all six showed bleeding of the uterine tissue after incision into the serosal surface. Histology revealed presence of endometrial tissue and myometrium but with patchy lymphocytic infiltration.

In the second experiment, which was carried out by our group, we used either CyA (IV) or tacrolimus (orally) in combination with prednisone (IV) to study their effect on the rejection of allotransplanted uteri. We found that the levels of CyA fluctuated during the treatment but tacrolimus levels remained fairly stable [140]. In both groups inflammatory changes were seen in comparison to auto-transplanted controls.

In 2009, Prof. Tzakis' group published an experiment in which 10 mini-swine were transplanted with uterine allografts [128]. The surgical model was a heterotopic transplantation of the graft with a vascular pedicle that included the bilateral uterine vascularization up to the aorta and vena cava. The animals were given tacrolimus IV for the first 12 days and 20 mg/kg/day of methyl prednisolone for a month. Then, maintenance immunosuppression was achieved with oral cyclosporine-A (10 mg/kg/ day). The animals were followed

up for a median time of 5.6 months (range 0.2 to 11.8). Six animals presented rejection episodes that were treated with steroids (10 to 20 mg/kg/day) and increased doses of oral cyclosporine [128]. After the first rejection episodes, methylprednisolone was kept at a dose of 10mg/kg/day for three months and no subsequent rejection episodes were registered.

Despite all of the experiments done in allogeneic UTx, it was not until 2006 [100] that the rejection process of the transplanted uterine graft was fully described in detail, which also proved the interaction between the donor cells and the recipient immune system triggers different immune responses that lead to rejection, like that seen in any type of allogeneic transplantation. Racho et al. and Groth et al. described in detail a time-dependent manner the effector mechanisms of uterine rejection in the allogeneic mouse UTx models [100, 101]. Using a combination of BALB/c donor mice and C57BL/6 recipient mice with no immunosuppression, they described how rejection starts by neutrophil and CD8+ lymphocyte infiltration of the myometrium on day 2 after transplantation and spreads to the endometrium from day 5. CD4+ lymphocytes only increase transiently on day 5 after transplant and CD19+ cell density remains low throughout the rejection process. The intensity of the rejection process can vary between species, as shown in two of our experiments. Rejection after fully allogeneic UTx between Brown Norway rat donors and Lewis rats recipients was successfully controlled with tacrolimus at 0.5mg/kg/day [97], while high doses of cyclosporine-A only partially suppressed rejection in a semi-allogeneic UTx mouse model (C57BL/6 mice were recipients of uteri from F1 hybrids -C57BL/6xCBA/ca donors) [141].

During the writing of this thesis we performed two sets of experiments involving allogeneic UTx in baboons. Both of them involved strong immunosuppression protocols similar to that used in composite tissue transplantation, including induction with antithymocyte immunoglobulins and corticoids, and maintenance with corticoids, tacrolimus and mycophenolate

mofetil [71, 142]. In the first experiment, 18 animals (*Papio anubis*) were allocated to receive no immunosuppression (Control group, n=4), tacrolimus in monotherapy with target serum levels of 5-10 ng/ml (TAC group, n=4), or the full immunosuppression protocol (FULL group, n=10) [142]. All animals with evaluable uteri presented variable degrees of rejection despite the immunosuppressive medication, with severe and total necrosis seen in all groups [142]. In the second experiment, six baboons (*Papio hamadryas*) received uteri from six mismatched donors [71]. Afterwards, they received the full immunosuppression protocol (see above). Variable degrees of rejection were seen in 5 out of 6 of the animals and only one of them kept an intact uterus [71]. One episode of acute rejection was controlled by increasing the dose of tacrolimus. The common problems in both experiments which might explain the adverse outcomes were that (1) oral immunosuppression was needed in much higher doses than in a human setting which is accompanied by highly variable serum levels of the immunosuppressants, and (2) Immunosuppression-related infections such as cytomegalovirus (CMV) resulted in very high mortality rate of 50% in one of the studies [71], and this would not be expected in a human setting.

4.2.5 Resistance to cold ischemia in uterus transplantation

A critical period in any kind of organ transplantation is the ischemic period, which is the time when the organ is disconnected from any kind of vascular supply. Absence of oxygen during ischemia results in energy depletion [143, 144]. However, most of the effector mechanisms of ischemic damage take place during reperfusion of the organ when toxic metabolites are flushed out of the organ. There are several interconnected mechanisms related to ischemia-reperfusion injury involving vasoconstriction [145], production of radical oxygen species [146, 147], increase of complement factors [148], and inflammatory cytokines [149], along with invasion of inflammatory cells [150]

and cell death [151, 152]. Subsequently, this early post-ischemic inflammatory response causes the maturation of immature dendritic cells in the transplant to become potent initiators of the adaptive immune response in the recipient [153, 154]. This influences the process of rejection, with an increased frequency of acute rejection events [155, 156], augmented chronic rejection [157], and delayed graft function [158].

The term *warm ischemia* is used to describe ischemia under normothermic conditions, and *cold ischemia* describes the period when the organ is chilled to around +4°C. In a transplant setting there is a first warm ischemic period during organ retrieval, from the time of vascular cross clamping until cold perfusion commences. This period is usually fairly short and seldom extends more than 3-5 minutes. The second warm ischemic period occurs after cold ischemia, during vascular anastomosis. The first warm ischemic period occurs in the donor at body temperature, and this period is generally more harmful to the organ than the second warm ischemic period when there is partial protection by preservation solution and when there is only a gradual increase in organ temperature of the organ, during which this ischemic period is situated in the recipient [159]. To avoid confusion, the terms first warm ischemia (donor) and second warm ischemia (recipient) have been suggested [160].

Cold ischemic storage, which is the period after flushing with a cold protective buffer, is the most commonly used method for minimizing the damage caused by ischemia during the transplantation process. Hypothermia decreases tissue metabolism by around 50% for every 10°C decrease in temperature [161]. The preservation solutions used for flushing and cold storage are designed to provide similar physiological conditions as in the blood in terms of pH and osmotic pressure [162].

It has also been acknowledged that various organs differ markedly in their capacity to withstand the harmful effects of hypothermic ischemia. For example, maximum recommended cold ischemic time is around 6 hours for the

heart and around 36 hours for the kidney and the pancreas. Experience regarding the resistance of the uterus to cold ischemia will be reviewed in the following paragraphs. Only experiments performed in the syngeneic or auto-transplantation models will be included to avoid the confounding factor of rejection. Avascular transplantation experiments will not be reviewed due to the remoteness of this experimental setup to the clinical reality.

-Mouse: In the initial mouse model of syngeneic, heterotopic uterine transplantation by vascular anastomosis [90], the uterus was flushed in situ with cold heparinized saline xylocaine. The cold ischemic time was about 35 minutes, and this was followed by second warm ischemia during vascular anastomosis that lasted for about 50 minutes. The graft survival was about 90% [90]. To test the limits of cold ischemic preservation, the mouse uterus was cold-stored in University of Wisconsin (UW) solution for 24 hours or 48 hours [91]. Uteri that had been stored ex vivo for 48 hours became necrotic after transplantation, but those preserved for 24 hours looked morphologically normal two weeks post–transplantation, and their subsequent functionality was demonstrated by the fact that 83% of the animals implanted embryos after transfer and later delivered normal offspring [91]. The advantages of the mouse model are that inbreeding diminishes the potential effects of factors other than ischemia on the histological findings. It also allows studying functionality and not only histology. On the other hand the uterine size of the mouse is much smaller than that of the human uterus, and this may be a factor to take into account when extrapolating the results to the human setting.

-Rat: Using the rat syngeneic model of heterotopic transplantation between inbred Lewis rats [133] rather than the surgically much more difficult mouse model (see above) allowed improved reproducibility and decreased operator dependency of this UTx model. In this model, the uterus was flushed in situ with 2 ml of ice cold heparinized Ringer Acetate supplemented with xylocaine

until uterus blanching occurred. Subsequent cold ischemia was performed for 60 minutes and the duration of warm ischemia (donor) was around 90 minutes. In these syngeneic uterine grafts, survival was 80% and only mild oedema and a slightly higher neutrophil count within the grafted uterus were noted, as compared to the native uterine tissue [133].

-Pig: Two studies assessed the viability of the uterus after ischemia-reperfusion, with conflicting results being reported. In the first study, Dr. Richard Smith's group performed measurements of oxygen saturation, and Doppler ultrasound measured blood flow on the graft after cold storage of the uterus ex vivo for 1 hour in UW/Celsior solution [3]. In another experiment performed by our group, uteri were kept for 1 hour in cold Ringer Acetate [94] ex vivo before auto-transplantation. While Doppler perfusion index and oxygen saturation measurements suggested satisfactory tissue perfusion in the first study [3], the results of the second study indicated that less than 20% of the uteri were correctly reperfused [94]. The explanation for such poor results in the latter study may have been the use of Ringer Acetate for cold preservation, rather than a proper preservation solution. A contributing factor may also be the rather long duration (1-2 h) of second warm ischemia during re-anastomosis [94]. Although analyses of the venous effluents of the properly reperfused uteri showed that blood gasses and lactate concentrations normalized after 60 minutes and that the levels of thiobarbituric acid-reactive species (indicators of oxidative stress) were normal [94], histology demonstrated a notable inflammatory response.

-Sheep: In our sheep model of autologous uterus transplantation [93], two solutions for cryopreservation were compared. Uteri were perfused in situ with either Ringer Acetate or the preservation solution Perfadex and then cold stored ex vivo before auto-transplantation. The cold ischemic time was about 70 minutes and the second warm ischemia period, during vascular anastomosis, was about 60 minutes. During reperfusion (3 h), blood samples were taken from

the uterine vein. Parameters related to glucose metabolism, oxidative stress and pH levels of the uterine venous blood normalized within 30 minutes in 70% of the auto-transplanted uteri [93]. An increase in neutrophil density within the uterine tissue was also found, and use of Perfadex partially avoided such infiltration.

-Human: Experiments to minimize ischemia-reperfusion injury during human uterus retrievals (n=7) from multi-organ donors [83] used heparin (30,000 IU) IV just before retrieval with flushing in situ with cold UW solution through the femoral arteries until the effluent was clear. The pelvis was filled with ice slush and the vagina was then divided. The duration of warm ischemia (donor) with this retrieval procedure was minimal. The uterus was stored in cold ischemic conditions for 12 hours. There were no signs of morphological alterations during the 12-hour interval of cold ischemia when examined at the light microscopy level [83]. However, it is questionable whether any signs of cellular damage would be evident at the light microscopy level after such a short interval of ischemia and with no reperfusion. In a similar setting of multi-organ retrieval, the group of Limoges analyzed uteri stored in Celsior at 4°C for 24 hours [132]. Histology studies did not find major morphologic changes after 24 hours of cold ischemia, and apoptosis was rare [132]. Another study performed by our group, used electron microscopy to assess the viability of cold-stored pieces of human myometrium. Small tissue samples of human uteri were subjected to cold (4°C) ischemia (6 and 24 h) in Ringer acetate (RIN), the intracellular-like UW solution, or the extracellular-like Perfadex solution (PER). Signs of hydropic degeneration and nuclear chromatin changes were detected by electron microscopy after 24 hours of storage [96], but analyses of contractility, ATP and protein concentrations showed that human myometrial tissue remains vital through at least 6 hours of cold preservation in UW or Perfadex [96].

4.2.6 Resistance to warm ischemia in uterus transplantation

There are only two experiments evaluating the effects of warm ischemia on uterine viability after UTx. In an experiment carried out by our group, syngeneic UTx was performed between Lewis rats. The animals were synchronized to avoid the possible effects of the estrous cycle on the distributions and densities of the uterine leukocytic subpopulations, since alterations in these may be an indication of ischemic damage with secondary inflammatory changes. Each recipient rat was allocated to receive the uterus after a standard warm ischemia time of 70 minutes or after an extended warm ischemia time of 300 minutes. Uteri were evaluated on day 3 and day 6 post-transplantation by visual inspection, examination of vessel patency and histological examination. The extended warm ischemia time negatively affected tissue viability, with 4 out of 10 animals presenting macroscopic necrosis of the graft.

The great tolerability of the uterus to warm ischemia was also tested in the sheep, when auto-transplantation of both the uterus and ovary was conducted for fertility purposes [95]. Even with warm ischemia extending over 3 hours, the uterus was still able to bear normal pregnancies.

It is difficult to predict the length of warm ischemia during a complicated case of human UTx, but we know that warm ischemia in uneventful cases can last around 80 minutes [163]. Although the principles of any kind of solid organ transplantation lead us to reduce the warm ischemia times as much as possible, extended warm ischemia times up to 3 hours 20 minutes have been described in a liver transplantation in the human setting [164].

4.2.7 Fertility after uterine syngeneic and auto-transplantation

The aim of any organ transplantation is to re-establish function of the non-functioning/non-existing organ. Therefore pregnancy with live and healthy

offspring is the only and final end-point of UTx. It is important to emphasize that when this PhD research started, data on births after UTx were limited to syngeneic or auto-transplantation experimental models.

-Mouse: In the year 2002, the first pregnancy after UTx was reported in a mouse model [90]. The transplantation was end-to-side between the vena cava and aorta of the recipient. The transplant was heterotopic, which means that the uterus was transplanted to a place different from the native pelvic location. In this model the native uterus of the recipient was left in place. The cervix of the heterotopically transplanted uterus was kept intra-abdominally to prevent ascending infections through the cervix of the graft. One mouse underwent embryo transfer. Since the complete uterine graft was positioned intra-abdominally, trans-abdominal embryo transfer was performed via a midline laparotomy using thin Pasteur pipette, which was inserted through the myometrium. Out of the six transferred blastocysts, three were placed inside the native uterus and three inside the graft with pregnancy evaluated 10 days after embryo transfer. The results were three fetuses of normal size in the native uterus and one fetus in the transplanted uterus, which in addition exhibited an absorbed pregnancy. No live offspring were obtained, however, this was not part of the study design as the experiments were ended halfway through pregnancy. In subsequent experiments, our group demonstrated live offspring for the first time using the same model with the minor modification [89, 91] that the cervix was connected at a cutaneous stoma of the lower abdomen. Thus, endometrial secretions were able to drain from the uterine cavity. Again, the native uterus was left in place to act as a control. The pregnancy rates were similar in the native (75%) and in the grafted uterus (66%) of the transplanted animals [89]. Importantly, the median numbers of fetuses in the pregnant animals were similar in the native and transplanted uteri (4 pups). As mentioned in the paragraph “Characterizing resistance to cold ischemia in uterus transplantation”, cold ischemia up to 24 hours did not negatively

influence the capability of a transplanted uterus to carry a normal pregnancy, with healthy live offspring obtained in 5 out of 6 transplanted animals [91].

The offspring obtained from the experiments described above were phenotypically normal. There was no difference in birth weight or length of animals, or in placental weights between the groups. Weight trajectory over 8 weeks was also similar between pups born from native and transplanted uteri [89].

-Rat: Heterotopic UTx in the human would probably present problems related to anatomical stability of the uterus if placed outside the pelvis. This is why an orthotopic model had to be developed in the rat. The first aim of the research project was the development of such a model, which was carried out within the experiments of this dissertation. Further details will be given in the methods and results sections.

-Sheep: The sheep was the first large animal model in which fertility was reported after UTx, although available data at the time of this research project were restricted to a single study. This was an auto-transplantation study in which the same animals were donors and recipients [95]. On analogy with the rodent model, only one horn of the ovine bicornuate uterus was transplanted in the procedure [87, 93, 95]. The graft consisted of the common uterine cavity with one uterine horn and also the ipsilateral oviduct and ovary. This also allowed for minimal surgical trauma to the oviduct, which is important for permitting natural conception. Vascular anastomosis of the anterior portion of the internal iliac artery, the utero-ovarian vein, and an aortic patch with the ovarian artery were accomplished on the external iliac vessels. Spontaneous cyclicity was evidenced and five animals were subjected to natural mating some months after transplantation. They were compared to five control animals not undergoing any surgery at all. All control animals mated and became pregnant with normal offspring. Four of the auto-UTx sheep mated, and pregnancies to full term were seen in 3 out of 5 animals [95]. One of these

pregnant ewes showed contractions compatible with the initiation of labor, but they disappeared after 24 hours. A caesarean section was performed and a 360°-rotated uterus containing a twin pregnancy was found [95]. The site of the torsion was the lower segment, and both lambs died. Uterine torsion at labor is a well-known complication in sheep, but it may well be that inadequate fixation of the transplanted uterus contributed to the torsion. In this study [95], sizes and weights of both lambs and placentae did not differ between the groups.

-Non-human primates: The higher non-human primates belonging to the group old world monkeys, such as the baboon and the macaque, have a simplex uterus and a vascularization pattern and a menstrual cyclicity that is close to that of the human [165]. This is why these species have been involved in UTx research [73, 88, 131, 166, 167]. Only two studies using primates have sought pregnancy after transplantation [73, 167].

In an auto-transplantation model using the olive baboon (*Papio anubis*), we transplanted the uterus together with the ovaries and Fallopian tubes, the main trunks of the internal iliac arteries, and extended dissections of the ovarian venous pedicles, including patches of the vena cava and left kidney vein [73]. At back-table preparation, the smallest iliac artery end of the graft was coupled end-to-end to the contralateral posterior branch of the internal iliac. At transplantation, this construct was coupled end-to-end to the proximal remnant of the internal iliac artery and the venous patch to the external iliac vein. The animal survival was 100%, while 80% of the animals resumed cyclic hormonal patterns and 60% of the animals resumed regular menstruation [73]. However, no pregnancy occurred despite breeding attempts over several months. Tubal blockage was later seen at post-mortem analysis [73]. Because of these results, our subsequent experiments in the baboon UTx model did not include the Fallopian tubes as part of the graft. Although IVF and embryo transfer has been described in baboons, it requires good embryology laboratory facilities and its efficiency is quite poor [168].

The cynomolgus macaque has also been used for autologous UTx experiments, being the first and only primate species in which live offspring have been reported [167]. An autologous UTx including unilateral Fallopian tube and ovary, and involving bilateral uterine artery anastomosis to external iliac arteries, was performed. Venous outflow was through one deep uterine vein and the contralateral ovarian vein, which were coupled end-to-side to the external iliac veins. Natural mating three months after transplantation resulted in a pregnancy that developed normally until placental abruption occurred 143 days after mating. A live offspring was delivered, but resuscitation of the neonate was not performed due to ethical issues [167]. No major malformation was evidenced in the newborn.

4.3 Immunosuppression

4.3.1 Principles of immunosuppression and rejection in allogeneic transplantation

4.3.1.1 Types of organ transplantation

There are four different types of organ transplantation procedures, depending on the genetic proximity of the donor and the recipient [169]:

-Autologous (auto-)transplantation: refers to transplantation of tissue or organs from one individual to the same individual.

-Syngeneic transplantation: refers to transplantation between two individuals that are genetically identical (e.g. identical twins) or that are almost genetically identical (e.g. inbred strains of animals (mouse/rat)).

-Allogeneic transplantation: refers to transplantation between two individuals that are genetically different. In a clinical setting, solid organ transplantation (e.g. kidney, liver or uterus) is usually done between two genetically different individuals of the same species.

-Xenotransplantation: refers to transplantation between individuals from different species.

4.3.1.2 Mechanisms of recognition of foreign antigens

Recognition of foreign molecules by T-lymphocytes occurs when the major histocompatibility complex (MHC) of APC (antigen-presenting cells) presents antigens to the cell membrane-bound TCR receptors of the lymphocytes [169]. There are two main pathways of allorecognition (Figure 3): 1) direct allorecognition: when T-lymphocytes from the recipient interact with intact MHC molecules from APC of the donor (foreign MHC); 2) Indirect

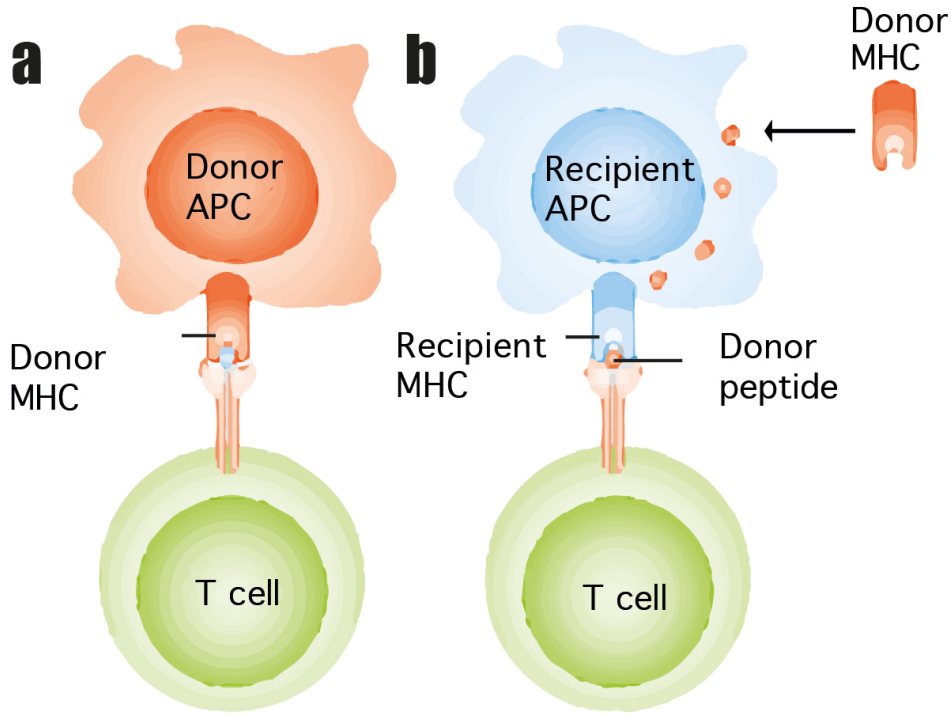


Figure 3. Mechanisms of allorecognition. A) During direct allorecognition, which is unique to transplantation, T cells recognize intact allogeneic MHC molecules (together with bound endogenous peptide) on the surface of donor antigen-presenting cells (APCs) in the graft. Direct recognition by T cells of donor alloantigens on donor dendritic cells leads to full T-cell activation and graft rejection. By contrast, direct allorecognition by T cells of intact MHC molecules expressed on the surface of parenchymal graft cells that lack co-stimulatory activity might render the T cells refractory to further stimulation. This can induce a state of T-cell anergy. B) During indirect allorecognition, which is analogous to the T-cell response to protein antigens, alloantigens are recognized as linear peptides in the context of recipient MHC class II molecules after they have been processed and presented by recipient APCs.

allorecognition: when antigens from the donor are processed by APC from the recipient and are then subsequently presented to T-lymphocytes through the native MHC molecules of the recipient's APC. The response of the recipient's

immune system to foreign antigens or a foreign MHC results in effector mechanisms, including cytokine production, complement activation, lymphocyte and endothelial activation, and subsequently cell-mediated and antibody-mediated cytotoxicity [169].

4.3.1.3 Clinical classification of rejection

From a clinical point of view, rejection responses are generally classified into three groups, depending on the interval between transplantation and clinical manifestation of rejection [169]:

- hyperacute rejection occurs within minutes/hours after transplantation
- acute rejection usually occurs after the first week post-transplantation
- chronic rejection occurs several months after transplantation

Although there is not a specific relation between the immunological mechanism causing rejection and the time of onset of the clinical signs, hyperacute rejection is usually mediated by preformed antibodies and direct allorecognition of MHC molecules [170]; acute rejection is mediated by T-cell cytotoxicity and macrophage recruitment [171]; and chronic rejection appears to be antibody-mediated and is characterized by an extended fibrosis of the parenchyma and intima proliferation of the vessels of the graft [172].

4.3.1.4 Immunosuppressants

To avoid organ rejection, multi-drug immunosuppression protocols are used.

*Induction protocol: Used during the perioperative period to eliminate circulating cytotoxic lymphocytes [173-175]. The induction protocols usually include one or two administrations of polyclonal antibodies (antithymocyte globulin) or monoclonal antibodies (basiliximab, daclizumab), together with corticoids for a short time. This is followed by a calcineurin-inhibitor (see below), which is usually combined with an antiproliferative (see below) agent during the initial months.

*Maintenance immunosuppression: long-term immunosuppression is required to avoid acute episodes of rejection as well as chronic rejection. Maintenance immunosuppression is achieved with a combination of two or three of the following groups of drugs [175]:

-Calcineurin inhibitors (CINs)(cyclosporine, tacrolimus): CINs bind to the calcium-dependent serine-threonine-protein-phosphatase, calcineurin (CaN), together with the so-called tacrolimus binding proteins (FK506BPs or FKBPs) or immunophilins. The immunosuppressive effect of CINs is mainly achieved through the interaction of CaN+FKBP12+tacrolimus or CaN+Immunophilin-A+cyclosporine-A [176]. Such interactions block the phosphatase activity of CaN, and prevent dephosphorylation of the transcription factor NFAT, which inhibits the expression of interleukin (IL)-2, thereby inhibiting the proliferation of activated T-lymphocytes. Apart from this, CINs can affect other biological processes by binding different types of immunophilins that are involved in modulating the transcriptional activity of different steroidal receptors, and also by modulating the activity of the uterine natural killer cells (uNK) that are abundant in the endometrial stroma.

-mTOR inhibitors (sirolimus, everolimus): Sirolimus is a macrolide antibiotic produced by the fungus Streptomyces hygroscopicus. Everolimus is the major metabolite of sirolimus. These inhibitors block downstream mTOR pathways regulating cellular metabolism, growth, and proliferation. Their immunosuppressive effect is mainly achieved by inhibition of T-cell proliferation and proliferative responses induced by several cytokines [9].

-Antimetabolites (azathioprine, mycophenolate mofetil-MMF-): Azathioprine acts as a pro-drug for mercaptopurine. It mainly acts through the inhibition of the enzyme amidophosphoribosyltransferase, blocking DNA synthesis by interfering with purine metabolism. Thus, it most strongly affects proliferating cells, such as the T cells and B cells of the immune system, but also proliferating bone marrow cells, leading to medullar aplasia [177]. Their

metabolites can also block other enzymes involved in different nucleic acid metabolism.

MMF is the precursor of mycophenolic acid, an immunosuppressive compound isolated from the fungus Penicillium glaucum that inhibits inosine monophosphate dehydrogenase, an enzyme needed for the growth of T cells and B cells [178].

-Corticosteroids (prednisolone, methyl-prednisolone): These are synthetic derivatives of the natural adrenal steroids. Prednisone-derived corticoids display a mixed glucocorticoid and mineralocorticoid profile (mainly the former). Like the natural corticoids, they act through the cytosolic corticoid receptor, modulating a myriad of biological processes that result in the interference of inflammatory response, and humoral and cellular immune responses [179].

4.3.2 Side effects of immunosuppression on the patient

The combination of drugs with different mechanisms of action gives potent immunosuppressive effects with low dose-related toxicity [180]. However, immunosuppression is not free of side effects. The use of immunosuppressants has been related to the development of comorbidities such as kidney disease [181], diabetes [182], infections [183] and malignancies [184]. Such comorbidities are often a direct consequence of the use of immunosuppressants and can compromise directly or indirectly the survival rates of the patients.

-Chronic kidney disease (CKD): It is a very common complication after any kind of solid organ transplantation, regardless of whether the transplantation involves the kidney itself. It has been reported that up to 50% of patients transplanted with solid organs develop impaired kidney function [185]. There are different factors that can influence the progression of the histological and functional damage [186]. Many of these factors cannot be modified, like the nature of the organ transplanted, the age of the patient, the female gender, or

the use of calcineurin and mTOR inhibitors. Calcineurin inhibitors induce vascular obliteration, focal hyalinosis of small renal arteries and arterioles, global or segmental glomerulosclerosis, tubular atrophy, and striped interstitial fibrosis, resulting in higher CKD rates than other immunosuppressants [187].

The presence of comorbidities, especially impaired kidney function before transplantation, can also play a role in the development of CKD post-transplantation. It should be noted that UTx patients are healthy despite their infertility, and that a proper preoperative screening allows for the identification of patients at risk.

-New onset diabetes after transplantation (NODAT): It is a common condition affecting up to 24% of transplanted patients within 36 months after transplantation [188]. The incidence of NODAT depends on the definition used and the presence of other co-morbidities like obesity. NODAT has a severe impact on the graft's and patient's survival [182, 188] by increasing the incidence of cardiovascular events and infections [182]. Immunosuppressive agents can cause NODAT through different mechanisms, for example by inducing insulin resistance through decreased binding of insulin to insulin receptors [189] or by promoting gluconeogenesis [189], but they can also reduce the release of insulin by the Langerhans cells of the pancreas [190].

-Infections: Immunosuppressed patients are at a higher risk of developing infections than the general population [191]. It is difficult to assess the risk of infection in a given patient, which can change over the time [183], especially if there are modifications in the immunosuppressive therapy. The potential sources of infections include donor-derived infections, recipient-derived infections, nosocomial infections, and community infections [183]. Since infection is the main cause of first-year mortality in solid organ transplantation [192], it is of paramount importance to minimize the risk of infection by implementing active prophylaxis of the most common etiologies of infection. This should be done both to avoid unnecessary delays in diagnosis and to

effectively treat infections that can compromise the patient's survival. The immunosuppressed patient presents some particular features that make infection management different from that of the non-immunosuppressed patient. Most typically, inflammatory responses and seroconversion are usually impaired, which delay diagnosis, antimicrobial resistance is increased, and interactions between antibiotics and immunosuppressants are also very frequent, boosting toxicity of both.

-Malignancies: Transplanted patients have an incidence ratio of malignancies that is double that of non-transplanted patients [184]. For some specific sites like the lip, anus, vulva, and skin, the standardized incidence ratios (SIR) are increased more than 60 times [184] compared to general population. Factors that influence the post-transplant risk of malignancy are the length of exposure to immunosuppressive therapy, the intensity of therapy, and even the type of immunosuppressant used, with the highest risk associated with the use of calcineurin inhibitors, and the lowest risk with the use of mTOR inhibitors [193]. In composite tissue transplantation, in which healthy patients are transplanted, malignancies have also been reported [194].

4.3.3 Immunosuppression and pregnancy

Recommendations issued from the American Society of Transplantation held in 2005 [195] state that pregnancy could be considered one year after solid organ transplantation if rejection episodes have not occurred [195, 196]. At present, more than 15,000 births from transplanted women under immunosuppression have been reported without any noticeable increase in the rate of structural malformations. Increased risks of adverse perinatal outcomes have been described in voluntary registries [197, 198], but such voluntary registries are susceptible to selection bias. In a population-based study on pregnancy outcome in transplanted patients carried out in Sweden, higher rates of perinatal complications such as preeclampsia (OR: 6.5; 95%CI: 4.4-9.5), preterm birth

(OR: 13; 95%CI: 10-17), low birth weight (OR: 16; 95%CI: 12-20), and small for gestational age (OR: 6.4; 95%CI: 4.2-9.5) were also reported [199]. However, one of the most important findings of this study was that when births before and after transplantation were compared, the perinatal outcomes were found to be similar [199]. The interpretation was that the major cause of these pregnancy complications in transplanted patients is the organ disease that led to the transplantation procedure, and not the immunosuppressants or the transplantation procedure itself. In our group, we have recently carried out another population study in Norway (see paper #6#), involving 195 births after transplantation and 2260 birth before transplantation. In this study we were also able to compare perinatal results before and after transplantation within the same patient, and although the general results were similar to the previous Swedish study [199], we found lower rates of preeclampsia, preterm birth, growth restriction problems and perinatal mortality (See paper #6#).

Specific considerations must be made regarding the use of immunosuppression during pregnancy:

-Teratogenicity: some of the immunosuppressive drugs commonly used in solid organ transplantation like mycophenolate have teratogenic profiles [200] and should be withdrawn before pregnancy. Others, like azathioprine, have not been identified as a human teratogen, although anomalies have been reported in rodents exposed to the drug in utero [201], and it should therefore only be used if alternatives are not available.

-Pharmacokinetics: Physiological changes during pregnancy involve increased volume distribution and induction of different enzyme complexes that result in immunosuppressant altered metabolism [202]. These facts mandate that levels of immunosuppressive drugs be as low as possible and closely monitored during pregnancy.

4.4 ART in uterus transplantation

As mentioned in the previous paragraphs, we learnt from non-human primate models that the Fallopian tubes are not functional after UTx. Moreover, the inclusion of the oviducts would present a risk for ectopic pregnancy to occur, and such a pregnancy would be very difficult to surgically remove since adhesions after UTx may be extensive. Thus, a laparoscopic surgical procedure may not be feasible as an ectopic pregnancy may bleed profusely and necessitate acute surgery. These facts imply that IVF should be the treatment of choice before an UTx trial. Ovarian stimulation and oocyte pick-up could be performed after transplantation, but due to the complex surgery and possible egg and male factor infertility, it is reasonable to complete IVF before transplantation. In case of unsuccessful IVF, gamete donation could be discussed, but it is critical to have cryopreserved embryos or a large pool of mature oocytes before transplantation surgery. Potential candidates for UTx have several particularities that must be taken into account before any attempt of IVF:

-The lack of uterus will evidently cause an absence of menstrual bleeding. The lack of this physiological indicator of the stage of the ovarian cycle can be easily replaced by ultrasound monitoring of the follicular pool, progesterone level to confirm ovulation [203], and urinary LH ovulation kits [204]. A long protocol may be preferred in this group of patients to assure pituitary down-regulation before starting a classic ovarian stimulation in the follicular phase. Nevertheless we know from fertility preservation programs that ovarian stimulation regimens starting in the luteal phase can yield as many mature oocytes as a classic stimulation cycle and with similar fertilization rates [205].

Another implication of the lack of uterus is that FSH should be used with caution as an ovarian reserve marker, due to the imprecision in defining the early follicular phase. In the clinical routine of our group we prefer to use

ultrasound and AMH for this purposes due to their diagnostic performance [206].

-Single ET (SET) prevents adverse perinatal outcomes related to multiple pregnancy [207]. UTx is a clinical scenario where the risks of a multiple pregnancy are added to those of the immunosuppression and to the potential risks of the UTx surgery itself, and whose effects on the pregnant uterus have never been evaluated in the human.

Accordingly, multiple pregnancy should be avoided in UTx, and the best way to achieve this goal is to systematically perform SET. The overall pregnancy rate after SET transfer of embryos cryopreserved at day 2 was 27% in our clinical IVF program.

-Anatomical and functional particularities related to the ovaries. In MRKH patients, some anatomical variation of the ovaries may exist, including unilateral ovarian agenesis and bilateral gonadal streaks [42, 208]. Extrapelvic location of ovaries, where these organs are positioned at a more cranial position and lateral to the external iliac vessels, has also been described in up to 16% of the MRKH patients [208]. Patients who have undergone a hysterectomy due to cervical cancer might also have undergone oophoropexy. In all of these situations, where the ovaries can be situated far from the vaginal vault, follicular aspiration for oocyte retrieval can be done through the abdominal wall for easier access [209]. From a functional point of view, differences between the typical and the atypical form of MRKH may exist. Thus, it has been reported that while patients with the typical form have significantly higher mean numbers of follicles, oocytes, MII oocytes, fertilization and cleaving embryos than patients with the atypical form [210], the clinical pregnancy rate is similar in surrogacy programs.

4.5 Surrogacy

The alternatives to UTx for the treatment of infertility are acceptance of the infertility, adoption, and gestational surrogacy. The latter is the only option among those three that provides a real possibility of achieving genetic motherhood.

Acceptance of infertility has for a long time been the only alternative for women without a functioning uterus. This may in some cases lead to a permanent fertility crisis, which may involve seeking a second opinion, (e.g. "reproductive tourism"), suffering, and psychiatric morbidity and accompanying costs for the infertile couple and society. Adoption is a satisfactory alternative for some couples, though many couples have difficulties accepting the fact that adoption invariably involves no genetic link to the child.

Gestational surrogacy involves the use of gametes from the infertile couple to generate embryos that are subsequently transferred into the womb of a surrogate mother who delivers the baby but who leaves the baby to the "commissioning parents". If the gametes originate from both commissioning parents, there is a genetic link between the baby and the commissioning parents (full surrogacy). If the gametes originate from only one of the commissioning parents (partial surrogacy), there is still a partial genetic link between the baby and the commissioning parents.

In the debate regarding uterus transplantation vs. gestational surrogacy, some authors have characterized surrogacy as a less expensive and less medically and technically advanced alternative [82, 211, 212]. This statement is unconvincing, however, especially considering that surrogacy is not allowed in many countries, thereby requiring those couples to resort to commercial surrogacy abroad, which increases costs tremendously. The cost of the full process can reach 120,000€ in some countries, according to surrogacy advocates (http://www.vientredealquilerespaña.es/precios).

The medical and psychological consequences of either method is difficult to assess, mostly due to the lack of large enough long-term follow-up studies on surrogates, recipients and their offspring, and for obvious reasons there is currently no such information available on individuals involved in UTx. From the scarce information available on surrogacy, the commissioning parents have been described as being happy with their choice, especially considering this their only chance of biological parenthood, although some reports have indicated minor problems when neither of the commissioning parents have a genetic link to the offspring [213].

The surrogate mothers have reported few problems, except for the occasional case when the surrogate has wished to keep the baby she has given birth to. Such situations, together with the possibility that the commissioning parents for some reason do not wish to or are unable to take care of the baby, are probably important reasons why gestational surrogacy has been legally prohibited in many countries. An important issue in this context is the commercialization of the surrogates, evidenced by articles in the popular press from South-East Asia reporting on wealthy westerners who have paid money to Indian clinics, which in turn allows potential surrogate mothers to spend the entire pregnancy in the clinic under hospital- or hotel-like conditions during which they are subject to antenatal care [214].

Several arguments have been presented against payment for surrogacy. These include insult to human dignity, the instrumentalization of the human body, potential exploitation of vulnerable women, and coercion of women. The ESHRE guidelines on Ethics and Law make a plea for altruism in gestational surrogacy, that is, no payment apart from necessary health care expenses during pregnancy, provided that such expenses are not covered by a health insurance system, as well as payment for loss of income for the surrogate during pregnancy. These guidelines also highlight the fact that there are no studies

done so far on the follow-up of children born as a consequence of gestational surrogacy.

4.6 Ethical aspects of uterus transplantation

Ethical guidelines for research and development towards human UTx were presented by FIGO in 2009 [84]. The procedure has also been discussed from a religious standpoint by representatives of the Catholic church [215], as well the Islamic religion [69, 216]. In our group, we promoted bimonthly meetings within the Ethics forum of Sahlgrenska University Hospital between 2010 and 2012. These meetings had the goal of preparing the submission of an ethics application to the institutional review board before the creation of a human UTx trial. In these meetings between representatives of the Ethics board of the Sahlgrenska University Hospital and clinicians and researchers of the local UTx group, the ethical aspects of this major innovative surgical procedure were thoroughly discussed, balancing risks and benefits and discussing the complex ethics around UTx. Importantly, all of the team-members, including doctors, nurses, practical nurses and secretaries, that would later be involved in the human UTx cases participated in these meetings. This was important for developing their personal ethical standpoints regarding the UTx trial before actively taking part in the clinical and research aspects of the study.

4.6.1 Risks of UTx

4.6.1.1 Surgical procedures

As with any surgical procedure, UTx theoretically carries some risk for both a live donor and a graft recipient. In many ways the procedure in the recipient is similar to kidney transplantation since the external iliac vessels are also used as a site of anastomosis. It must be kept in mind that the recipient of a kidney often has an end-stage renal disease (ESRD) with associated morbidity which is not likely to be associated with a UTx. However, complications seen after

kidney transplantation, such as lymphocele around the kidney (20%) which obstructs urinary outflow, may also occur in UTx. General complications associated with any kind of abdominal surgery like bleeding or infection can also occur, although this is extremely rare.

The risks for the donor (if a live donation is performed) are those associated with the hysterectomy procedure. Hysterectomy is generally very safe. The risks stated in the literature are as follow: bladder injury (0.9%), ureter injury (0.06%), intestinal injuries (0.6%), bleeding exceeding 500 ml (2.8%), and overall reoperation rate 0.8%. However, for obvious reasons there is no data on hysterectomy risk in healthy women with normal uteri. Moreover, a hysterectomy for live uterus donation would be a more extensive surgery than an elective hysterectomy for benign causes, since all the vascular pedicles have to be included. On the other hand, the uterus of a uterine-donating woman would not contain myomas or other benign pathologies, which may disturb the surgical field. These facts have to be taken into account when discussing the risks of hysterectomy at live uterus donation.

As always, it is imperative to inform the donors and recipients on the surgical risks involved, and a written, informed consent is mandatory.

4.6.1.2 Immunosuppression

The effects of immunosuppressants on the patient and the future fetus have been widely discussed in the paragraphs “Side effects of immunosuppression” and “Immunosuppression and pregnancy”, and they include diabetes, hypertension, infections and higher risk of neoplasia. Most of these effects appear after long-term use of immunosuppressants, and it should be kept in mind that once the graft has achieved its function (live birth of a healthy baby) these drugs can be stopped, removing the risk of adverse effects. Regarding the fetus, it seems clear that there is a slight risk of preterm delivery and low birth weight, as well as a risk of preeclampsia in mothers undergoing solid organ

transplantation [217]. As mentioned in the paragraph "Immunosuppression and pregnancy", it is important to note that such effect is probably due to the underlying disease motivating transplantation rather than the transplantation or the immunosuppression itself [199]. In this study, after birth, there was neither a decrease in kidney function in newborns from transplanted mothers, nor any detectable disturbance in the immune system of children exposed to immunosuppressants in utero, althougth the follow-up time was limited [199]. Taken together, human and animal data suggest that there may be an increased risk of miscarriage after UTx, but there is no data to support any increased risk of congenital malformations [102, 197, 199].

4.6.2 Societal, religious, and cultural attitudes to uterus transplantation

Society has the obligation to secure the welfare of its people with respect to both healthcare and economic growth. In many countries in the western world the population is aging and too few young people are contributing to the welfare system. Advanced maternal age increases the rate of infertility, and IVF is one method for securing individual welfare by improving treatment of this age group, and by guaranteeing new individuals able to contribute to the welfare of society. Other alternatives for promoting new inhabitants in society include adoption or surrogacy. As mentioned above, surrogacy is surrounded by religious, ethical and legal restrictions. In some countries surrogacy is legal, with some distinctions made if the surrogacy is commercial or altruistic [218]. The Catholic church is strongly against gestational surrogacy [219]. In Judaism it is believed that the duty of a family is to produce children [220], and so the Jewish community does not oppose gestational surrogacy. The Islamic interpretations state that only the one who has given birth to a child should be the child's mother, and that it is immoral to introduce a gamete from someone who is not the husband [216, 221]. There are, however, some differences

between different branches of Islam [222]. However, it seems that gestational surrogacy and adoption as we know it in the West is nearly impossible in most parts of the Islamic world [222].

4.6.3 Introduction of UTx in a public health system

The purpose of ethical reflections and analysis is to bring awareness to important value conflicts in the introduction of new medical methods. Moral principles that apply values and judgments to the practice of medicine (such as autonomy of the patient, beneficence not maleficence, and justice), often have conflicting effects where one principles opposes another. For example, the autonomy of the patient may be confronted by the principle of "nonmaleficence" if the patient asks for a treatment or procedure with higher risks than potential benefits according to established medical criteria. When it comes to the introduction of new treatments in a public health system, there are other principles not strictly related to the individual patient but rather to patients as a whole. These principles apply to priority and management of health politics and try to answer questions like: What can we do, what ought we to do, and especially, what can we afford to do considering the lack of resources for medical care and cure?

-The principle of human dignity: "All people are equal in dignity, regardless of personal characteristics and functions in society". This principle focuses on the value of a medical treatment for one patient or a specific group of patients. From an individual point of view, there is no doubt that UTx is ethically defendable, since the procedure gives a woman a biological possibility to give birth to a child of her own.

-The principle of need and solidarity: "Resources should be committed to the person or activity most in need of them". This principle still focuses on the value of a medical treatment for one patient or a group of patients. However, according to this principle this value must be compared with the needs of other

patients requiring medical treatments of different kinds within one medical speciality (vertical priority settings) and between different medical specialities (horizontal priority settings). Since UTx is not a life-saving treatment but rather a quality of life-improving treatment, it is not obvious that UTx is ethically defendable from a priority setting perspective.

-The principle of cost-efficiency: "When choosing between different fields of activity and measures, a reasonable relation between cost and effect, measured by improved health and improved quality of life, should be aimed for." This principle still focuses on the value of a medical treatment for one patient or a group of patients. However, this value emphasizes a reasonable relationship between cost and effect, measured by improvements in health and quality of life. Therefore, it is of great importance to undertake a close examination from a cost-benefit as well as a cost-effective perspective, and also to compare these options to the other available alternatives (surrogacy or even adoption).

5 AIMS

The general aim of this thesis was to investigate the effects of immunosuppressants on the reproductive system and offspring -in the context of uterus transplantation- using different experimental approaches. More specifically, the aim was to provide a proof of concept that allogeneic UTx under immunosuppression is a promising treatment that can lead to healthy viable offspring. This was accomplished by combining studies of physiology, molecular biology and epidemiology. The findings of these new experiments, combined with previously existing knowledge of UTx, led to a clinical trial investigating human allogeneic UTx as a treatment option for AUFI patients.

The specific aims were:

1. To assess the effect of the calcineurin inhibitor tacrolimus on the ovulation rate.
2. To assess the effect of the calcineurin inhibitor tacrolimus on the implantation rate.
3. To develop an experimental model of allogeneic UTx and immunosuppression that allows for natural mating with full-term pregnancy that may serve as a proof of concept of functional allogeneic UTx.
4. To compare the main physiological characteristics of the offspring delivered after allogeneic UTx to those of the offspring of control animals.
5. To determine the effect of transplantation in general, and immunosuppression in particular, on the obstetric outcome of human patients transplanted with solid organs.
6. To achieve live offspring following allogeneic UTx in the human.

6 JUSTIFICATION

When the studies included in this thesis were designed, no pregnancy had ever been described following allogeneic UTx, and consequently there was no information available on the potential effects of the procedure on the forthcoming offspring. Furthermore, there was conflicting information regarding the effect of transplantation on fertility; while some studies found an improvement of pregnancy rates after solid organ transplantation, others found an increased rate of miscarriage and adverse perinatal outcomes. The objective of the different studies presented in this thesis aimed to construct a body of knowledge on fertility after UTx that could be used to develop a clinical trial based upon solid experimental research. Without this knowledge, any new attempts at UTx in humans would have lacked the minimal justifications of preparation according to the IDEAL statement [8].

It must be pointed out that the order in which the papers are presented in this thesis is based on the date when the experiments were done and do not necessarily match the date of publication. The reason for this is that some of the experiments are based on data obtained from previous studies that were not published when the latter studies were designed. On the other hand, the decision to prioritize papers for publication was based on their potential impact on the scientific community. For example, data obtained from the implantation and ovulation experiments were used to design the experiments on pregnancy after allogeneic UTx, although the latter were published first due to their impact on the existing literature.

7 ROLE OF THE PhD STUDENT

-Paper #1# Cyclosporine-A but not tacrolimus decreases implantation rate in the mouse: **C. D-G.** was responsible of the design of the experiment, surgical procedures, evaluation of the results, and wrote the major part of the manuscript.

-Paper #2# Differential effects of the immunosuppressive calcineurin inhibitors cyclosporine-A and tacrolimus on ovulation in a murine model: **C. D-G.** designed the study, performed ovarian stimulation and surgeries, analyzed the results, and wrote the major part of the manuscript.

-Paper #3# Pregnancy after syngeneic uterus transplantation and spontaneous mating in the rat: **C. D-G.** took part in the animal experiments, collected the data, and wrote the major part of the manuscript.

-Paper #4# First report on fertility after allogeneic uterus transplantation: **C.D-G.** designed the study and performed surgeries, the follow-up of the animals, the retrieval of tissue/blood samples, data analysis, and wrote the major part of the manuscript.

-Paper #5# Pregnancy after allogeneic uterus transplantation in the rat: perinatal outcome and growth trajectory: **C. D-G.** designed the study, did the animal handling, and performed surgeries, tissue sample retrieval and synchronization of the foster colony. He also followed-up the pups issued from this experiment, analyzed the data and wrote the major part of the manuscript.

-Paper #6# Pregnancy outcome after maternal solid organ transplantation: nationwide population-based cohort study over four decades: **C.D.G.** was responsible for the design of the study, statistical analysis, and wrote the major part of the manuscript.

-Papers #7, 8, 9# These reports reflect different time points of the same clinical trial. #7# First clinical uterus transplantation trial: a six-month report; #8# Uterus transplantation trial: 1-year outcome; #9# Live birth after uterus

transplantation: **C. D-G.** participated in the design of the study, did surgery, obtained data, and participated in the writing of the manuscripts.

In addition to the above-mentioned papers, during the PhD program the candidate also obtained a FELASA C certificate to direct animal experiments and a Master's degree on Design and Statistics in Health Science from the Universidad Autónoma de Barcelona. He also participated in other research projects linked to different aspects of UTx and ovarian transplantation that resulted in the publication of multiple review papers and also the following original publications:

1: Morken NH, **Díaz-Garcia C**, Reisaeter AV, Foss A, Leivestad T, Geiran O, Hervás D, Brännström M. Obstetric and neonatal outcome of pregnancies fathered by males on immunosuppression after solid organ transplantation. Am J Transplant. 2015;15(6):1666-73.

2: Tryphonopoulos P, Tzakis AG, Tekin A, Johannesson L, Rivas K, Morales PR, Wagner J, Mölne J, Enskog A, **Díaz-Garcia C**, Dahm-Kähler P, Berho M, Zimberg S, Falcone T, Ruiz P, Olausson M, Brännström M. Allogeneic uterus transplantation in baboons: surgical technique and challenges to long-term graft survival. Transplantation. 2014 Sep 15;98(5):e51-6.

3: Olausson M, Johannesson L, Brattgård D, **Díaz-Garcia C**, Lundmark C, Groth K, Marcickiewizc J, Enskog A, Akouri R, Tzakis A, Rogiers X, Janson PO, Brännström M. Ethics of uterus transplantation with live donors. Fertil Steril. 2014 Jul;102(1):40-3.

4: Akhi SN, **Díaz-Garcia C**, El-Akouri RR, Wranning CA, Mölne J, Brännström M. Uterine rejection after allogeneic uterus transplantation in the rat is effectively suppressed by tacrolimus. Fertil Steril. 2013 Mar 1;99(3):862-70.

5: Johannesson L, Enskog A, Mölne J, **Díaz-Garcia C**, Hanafy A, Dahm-Kähler P, Tekin A, Tryphonopoulos P, Morales P, Rivas K, Ruiz P, Tzakis A, Olausson M, Brännström M. Preclinical report on allogeneic uterus transplantation in non-human primates. Hum Reprod. 2013 Jan;28(1):189-98.

6: **Díaz-García C**, Akhi SN, Martínez-Varea A, Brännström M. The effect of warm ischemia at uterus transplantation in a rat model. Acta Obstet Gynecol Scand. 2013 Feb;92(2):152-9.

7: Johannesson L, **Díaz-Garcia C**, Leonhardt H, Dahm-Kähler P, Marcickiewicz J, Olausson M, Brännström M. Vascular pedicle lengths after hysterectomy: toward future human uterus transplantation. Obstet Gynecol. 2012 Jun;119(6):1219-25.

8: Johannesson L, Enskog A, Dahm-Kähler P, Hanafy A, Chai DC, Mwenda JM, **Díaz-García C**, Olausson M, Brännström M. Uterus transplantation in a non-human primate: long-term follow-up after autologous transplantation. Hum Reprod. 2012 Jun;27(6):1640-8.

9: Milenkovic M, **Díaz-Garcia C**, Wallin A, Brännström M. Viability and function of the cryopreserved whole rat ovary: comparison between slow-freezing and vitrification. Fertil Steril. 2012 May;97(5):1176-82.

10: **Díaz-García C**, Milenkovic M, Groth K, Dahm-Kähler P, Olausson M, Brännström M. Ovarian cortex transplantation in the baboon: comparison of four different intra-abdominal transplantation sites. Hum Reprod. 2011 Dec;26(12):3303-11.

8 STUDY DESIGN OF THE EXPERIMENTS

In the following paragraphs the general design of the experiments included in this thesis are presented. Extended information on the methods used in each experiment can be found in the respective paper.

8.1 Paper 1- Assessment of the effect of calcineurin inhibitors on implantation

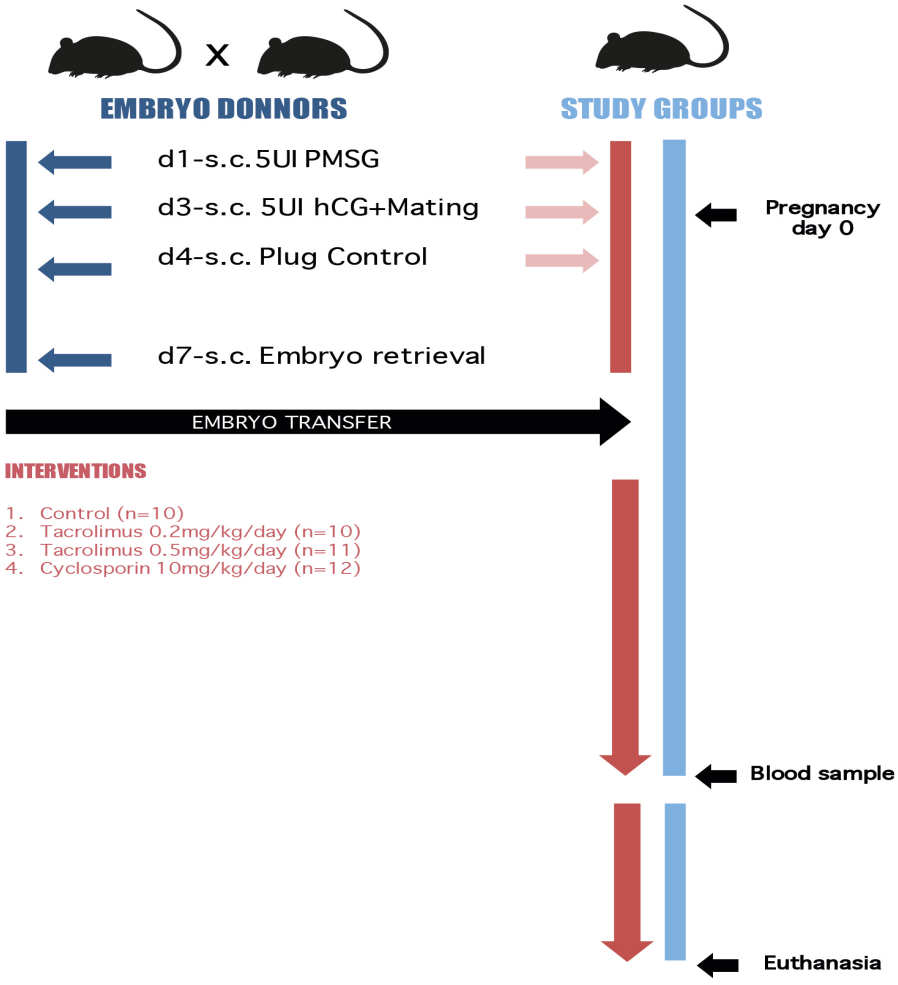


Figure 4. Schematic view of the design of the implantation experiment. Vertical lines represent time-lines (Breeders generating embryos in dark blue, study animals in red). Animal manipulations are marked with arrows.

Adult female B6CBAF1 mice (n=43) were randomly allocated to receive daily doses of one of the following immunosuppressant treatments: 1) tacrolimus 0.5mg/kg/day (TAC05 group); 2) tacrolimus 0.2mg/kg/day (TAC02 group); 3) cyclosporine-A 10mg/kg/day (CYA10 group); 4) normal saline 0.9% (Control group). After induction of pseudo-pregnancy, each animal underwent transfer of 14 blastocysts from the same strain (7 blastocyst per horn), and 7 days later implantation sites and fetal viability were assessed by direct inspection of the uterine cavities. Tissue samples were also taken for natural killer (NK)/placentation markers characterization by immunohistochemistry and RT-PCR. A schematic overview of the study design is shown in Figure 4.

8.2 Paper 2-Assessment of the effect of calcineurin inhibitors on ovulation

Immature Sprague-Dawley rats (21 days of age) were randomly allocated (15 animals/group) to receive one of the following immunosuppressive drugs: Group 1-0.9% NaCl (Control); Group 2-cyclosporine-A 10mg/kg/day (CYA10) and Group 3-tacrolimus 0.5mg/kg/day (TAC05). All animals were synchronized for ovulation at 25 days of age according to our standardized protocol for ovulation studies, which consists of administration of 10 IU of equine chorionic gonadotrophin (eCG) s.c. at 12:00 on day 25. Forty-eight hours later, the rats were administered 10 UI of human chorionic gonadotrophin (hCG), with ovulation occurring 10-15 h after hCG administration [223]. Twenty hours after inducing ovulation, the animals underwent euthanasia and the ovulation rates were assessed by counting the number of oocytes present in the Fallopian tubes. Blood samples and both ovaries were also harvested in order to perform leukocyte quantification and measurement of calcineurin inhibitor (CINs) levels in peripheral blood, and histological and molecular

markers of neutrophils and ovulation. A schematic overview of the study design is shown in Figure 5.

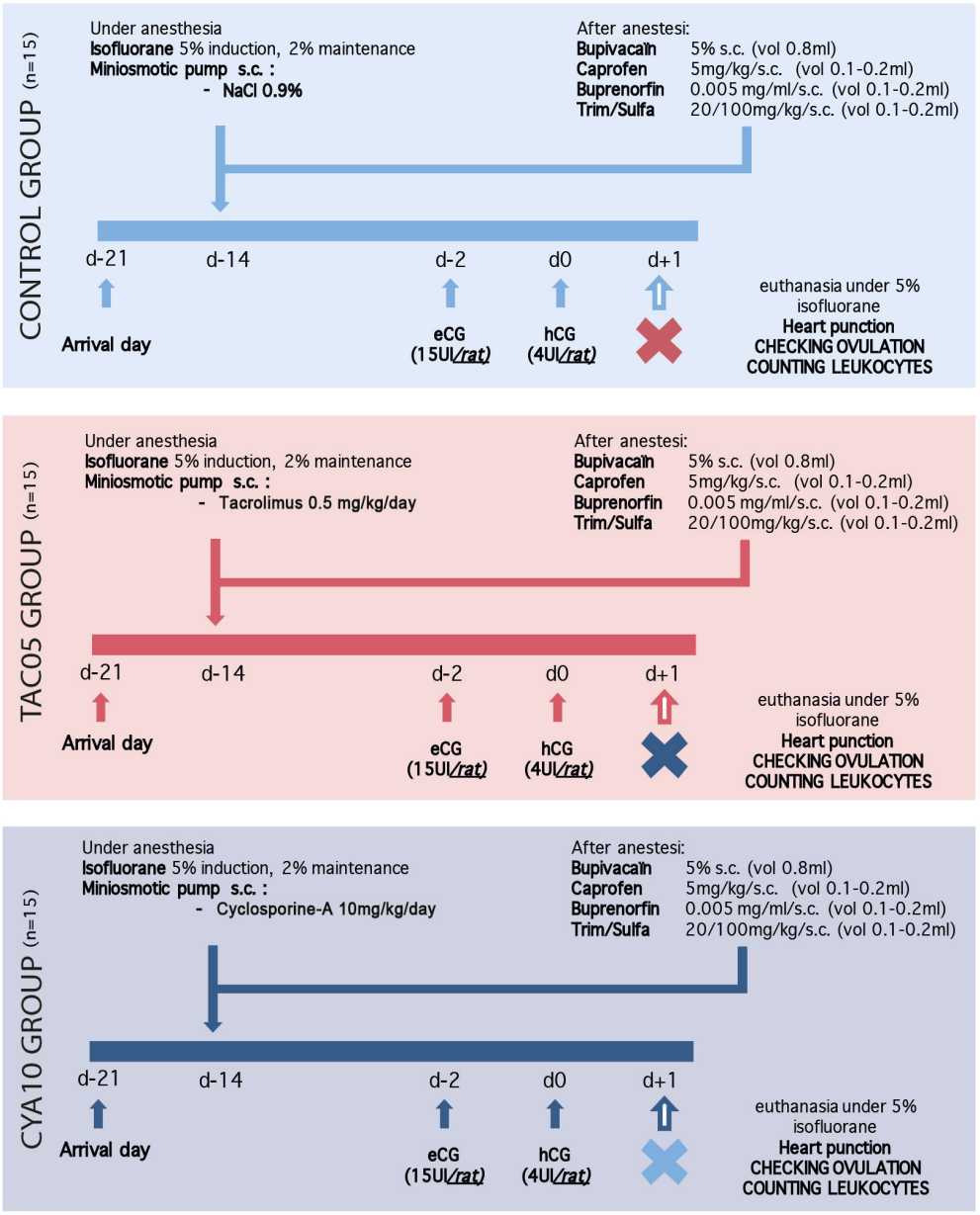


Figure 5. Schematic view of the design of the ovulation experiment. Animals were allocated to receive one of the interventions in a three-arm parallel design. Horizontal lines represent time. Manipulations of the animals are marked with arrows.

8.3 Papers 3 and 4-Development of an experimental model of allogeneic uterus transplantation allowing for pregnancy

The rat was chosen to develop an allogeneic model of UTx. As compared to our previous experience in the mouse, the rat offers a shorter learning curve for the surgery and a higher reproducibility between surgeons [133]. On the other hand, IVF procedures in the rat are difficult and are less effective than in the mouse [224]. For these reasons, it was decided that an orthotopic UTx surgical model that allowed for natural mating should be developed. Initially, uteri were transplanted between individuals from the same strain (syngeneic transplantation) to avoid the effect of the rejection on the outcomes of the surgery. Once the surgical model was developed, the findings were used to develop a model for the allogeneic setting with immunosuppression. This was done in order to carry out the fertility experiments under conditions similar to those expected in a clinical setting.

8.3.1 Development of the surgical technique (Paper 3):

Female virgin Lewis rats were randomly allocated for syngeneic uterine transplantation (UTx group; n=27) or removal of the left uterine horn (Sham group; n=19). In the donor, a graft was isolated which contained the right uterine horn, the common uterine cavity with the cervix, and the upper vagina plus a vascular pedicle including the arteries and veins from the right uterine vessels up to and including the right common iliac vessels. To allow for natural conception, an undisturbed uterine-tubal junction seemed to be a prerequisite. Thus anastomosis was performed to connect the major part of the uterine horn of the graft to a smaller upper part of the recipient uterine horn. Thus, the donor uterine horn was divided 7-8 mm from the Fallopian tube, after cauterizing the utero-ovarian pedicle at the same level. The uterus was stored in cold (4°C) Perfadex preservation solution (Vitrolife AB, Mölndal, Sweden) during

preparation of the recipient. In the recipient, a simple hysterectomy was performed, keeping a 7-8 mm segment of the upper portion of the uterus. The right common iliac vessels were dissected and the common iliac vessels of the graft were anastomosed to the recipient in an end-to-side fashion using two hemicontinuous 10-0 nylon sutures (S&T Microsurgery, Neuhausen, Switzerland) on each vessel (Figure 6, panel A). The vaginal cuff of the transplant was then sutured to the vaginal vault of the recipient and the uterine horn of the graft was anastomosed end-to-end (Figure 6, panel B) to the remaining cranial uterine segment of the recipient uterus.

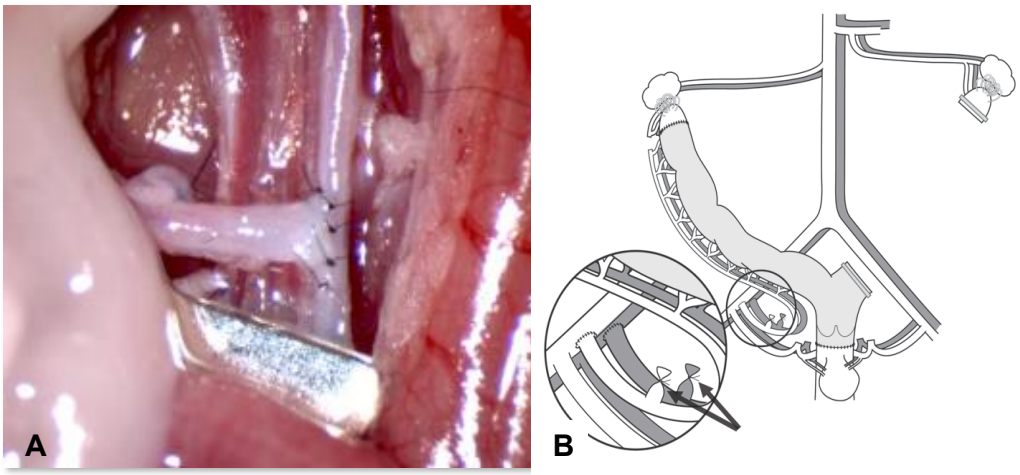


Figure 6. A) Detail of the anastomosis site on the common iliac vessels of the recipient. The internal iliac artery of the graft is attached with interrupted sutures to the common iliac artery of the recipient. B) Schema of the recipient surgery: The graft is colored in grey, the recipient in White; a detail of the anastomosis site is augmented in the circle.

Control animals were operated under the same conditions as the transplanted rats to remove the right uterine horn. No modifications were made on the remaining horn and only blood vessels and nerves coming through the left infundibulopelvic ligament were excluded.

After recovery after surgery, animals mated with males of proven fertility. The efficiency of the model was assessed by evaluating the reproductive performance and achievement of viable offspring.

8.3.2 Fertility experiments in an allogeneic setting (Paper 4):

Twenty adult Lewis rats were randomly allocated to one of the following groups: 1) Allogeneic UTx under immunosuppression (UTx-TAC group; n=9); 2) Sham surgery under immunosuppression (Sham-TAC group; n=5) and 3) Sham surgery without any immunosuppression (Sham non-TAC group; n=6). The surgical procedures were similar to those described in the previous experiments. UTx were performed between female virgin Dark Agouti rats (histocompatibility haplotype: RT1av1, RT2b, RT3a, RT7a)—donors—and female virgin Lewis rats (histocompatibility haplotype: RT1l, RT2a, RT3a, RT7a, RT8b)—recipients. The immunosuppression protocol consisted of continuous administration of tacrolimus by means of mini-osmotic pumps to achieve a final dose of 0.5 mg/kg/day. All animals were exposed to fertile males during one estrous cycle between 35 and 38 days after surgical intervention. Mating was confirmed and mated animals underwent cesarean section 17 days later to assess both the number of viable and the number of resorbed pregnancies.

8.4 Paper 5-Evaluation of the perinatal outcome and growth trajectory of the offspring issued from mothers transplanted from an allogeneic uterus

In this experiment, data from papers 3 and 4 were used to develop a different allogeneic UTx model, allowing for less aggressive immunosuppression. PVG female rats were randomly allocated to three different experimental interventions: (A) allogeneic UTx under immunosuppressive treatment with tacrolimus (UTx+TAC group, n=10), with Virgin Lewis rats (histocompatibility haplotype: RT1l, RT2a, RT3a, RT7a, RT8b) used as uterus donors and virgin Piebald-Virol-Glaxo (PVG) rats (histocompatibility haplotype: RT1c, RT2b, RT3a, RT6a, RT7a, RT8b) used as recipients; (B)

surgical removal of the left uterine horn under immunosuppressive treatment (Sham+TAC group, n=10); and (C) surgical removal of the left uterine horn without any immunosuppressive treatment (Sham group, n=10). In the groups receiving immunosuppression (UTx+TAC and Sham+TAC groups), animals were given tacrolimus at a dose of 0.4 mg/kg/day for 8 weeks, which was then tapered to 0.2 mg/kg/day during the remainder of the experiment. The female rats were introduced to males of proven fertility and the mating and pregnancy rates, numbers and sex of pups, and weights and perinatal mortality rate were recorded and compared.

Two breeding colonies were used as external control groups for weight: a) pups born from PVG females (BC; n=52), and b) and pups born from PVG females undergoing an identical immunosuppressive treatment as the experimental groups during pregnancy (BC+TAC; n=86). Comparisons were made between pups born from the three experimental groups and also between pups born from the UTx+TAC group in comparison to those of the breeding colonies. A schema of the study design is shown in Figure 7.

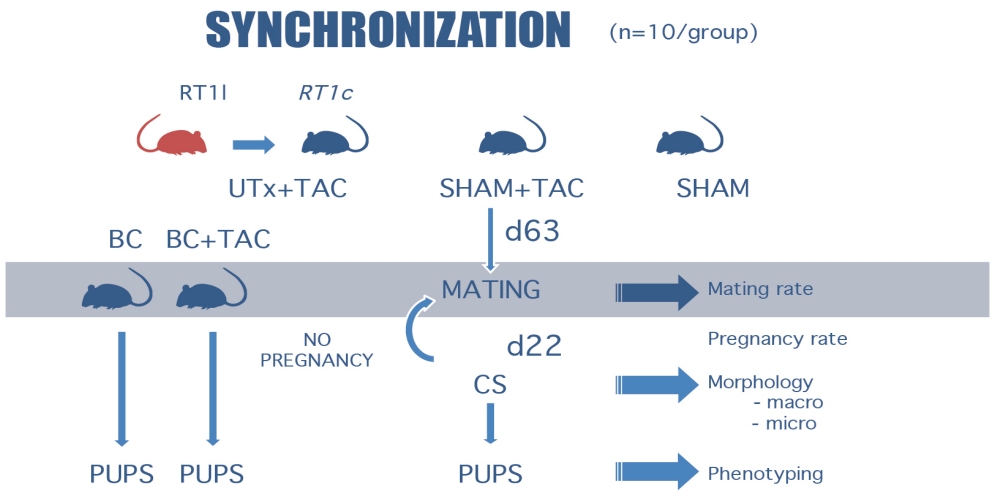


Figure 7. Schematic view of the study design. Three experimental groups were compared between them and also with two external breeding colonies, one receiving tacrolimus (BC+TAC) and the other one not (BC).

8.5 Paper 6-Evaluation of the perinatal outcome of pregnancies mothered by transplanted patients

In this study, in order to assess the risk of adverse perinatal outcome in pregnancies from transplanted mothers, we performed a cross-link cohort study between two large population databases that extended over a period of 40 years. The first database was the Medical Birth Registry of Norway (MBRN) and the second one was the Transplantation registry from Oslo University (TROU). Oslo University Hospital has since 1969 kept complete records of all transplanted patients in the country within the TROU. Both registries are compulsory. All records in the MBRN are matched with the files of the Central Person Register (unique identification number given to every Norwegian citizen or resident), and are the records of the TROU. Linkage between TROU and the MBRN was performed using this unique personal identification number. Information about type of transplanted organ and date of transplant were collected from the TROU, whereas data on pregnancy, delivery, perinatal complications and congenital malformations were obtained from the MBRN.

The analysis included all female patients that received a transplant during the period from January 1969 up to and including 2009. Twin pregnancies and transplanted males were identified to exclude any pregnancy emerging from couples with both parents being immunosuppressed, and thereby to exclude possible influences carried over from the male. Possible confounding factors for adverse perinatal outcome were controlled using multivariate regression analysis and propensity-scores.

Comparisons were made between transplanted patients and the general population but also within transplanted patients: pregnancies before and after

transplantation were compared list-wise between patients and paired-wise within the same patient.

8.6 Papers 7, 8, 9-Description of the first live birth after allogeneic uterus transplantation in the human

After the intense preclinical research described in the introduction of this thesis and also after the fertility experiments developed during this PhD project, a clinical trial in humans was approved by the Regional Human Ethics Committee of Sahlgrenska in 2012 and then registered as a clinical trial (NCT01844362). Permit was obtained to perform allogeneic uterus transplantation between live donors and recipients in ten patients. The inclusion and exclusion criteria are shown Table 2.

8.6.1 Preoperatory assessment:

The screening process included tests to assess the general medical health status, psychological health, status of infectious diseases and HLA and HLA-antibody screening. It was also designed to assess the suitability of the organ to be transplanted and to rule out the presence of pathology in the uterus potentially affecting the ability to carry a normal full-term pregnancy. The different parts of the screening process were performed by a multidisciplinary team including gynecologists, transplant surgeons, psychologists, immunologists, internal medicine doctors, anesthesiologists and radiologists.

An external transplantation board verified all the screening process and took the final decision on whether or not to include each independent donor-recipient pair.

Table 2. Inclusion and exclusion criteria

	RECIPIENT	DONOR
Inclusion	< 39 years	< 60 years
	Good ovarian reserve (AFC / AMH)	Previous normal pregnancy
	IVF (> 10 embryos cryopreserved)	Accomplished child wishes
Exclusion	BMI >30 kg/m2	BMI >30 kg/m2
	Presence of infectious/systemic disease	Presence of infectious/systemic disease
		Uterus with pathology compromising pregnancy (myomas <1cm are allowed)
		Major abdominal surgery

8.6.2 IVF before transplantation

A key step of the preoperatory assessment was to rule out any fertility problem up to fertilization in order to identify patients having a potential sterility background that for obvious reasons (the lack of uterus) should be excluded. All patients were treated using the long GnRH-agonist protocol to achieve a uniform hormonal situation before gonadotrophin treatment was initiated. The agonist treatment (Buserelin, Suprecur, 0.15 mg/dose; Sanofi Aventis AB, Bromma, Sweden) was administrated in the midluteal phase based on earlier cycle monitoring of estradiol (E2), progesterone (P4), luteinizing hormone (LH) and follicle stimulating hormone (FSH), usually 8 to 9 days after positive urine LH-testing (ClearBlue, SPD Swiss Precision Diagnostics, GmbH). Gonadotrophin doses for stimulation were determined based on age, BMI and AFC of the patient, and human menopausal gonadotropin (hMG) (Menopur, Ferring AB, Sweden) or recombinant follicle stimulating hormone (rFSH) (Gonal-f, Merck Serono, Europe, Ltd, UK) were used. Final follicular and oocyte maturation was induced by hCG injection (Ovitrelle, Merck Serono, Europe, Ltd, UK or Pregnyl, MSD, Sollentuna, Sweden) and oocyte aspiration performed 36 h later using follicle aspiration set with single lumen (Vitrolife

Sweden AB, Göteborg,). Oocytes were aspirated vaginally according to our routine method [225] using local anesthesia, combined with mild sedation and analgesia if needed. In cases when ovaries were not readily reachable via the vaginal route, oocytes were aspirated by transcutaneous puncture in local anesthesia and mild sedation. Fertilization was performed 4h after oocyte pick-up and normally fertilised and cleaved embryos were cryopreserved on day two, using the slow freezing method and Sydney IVF cryopreservation kit (COOK Medical, Limerick, Ireland) or in blastocyst stage using the cryotop vitrification kit (Kitazato, Dibimed Biomedical Supply S.L., Valencia, Spain).

8.6.3 Preoperatory preparation, surgery and postoperatory care

Preoperatory preparation, surgery and postoperative care are described in detail in paper number 7. Briefly, anesthesia was administered using a combined spinal-general anesthesia. Prophylactic antibiotics consisted of piperacillin/tazobactam and thrombosis prophylaxis was achieved with a combination of 5,000 IU of dalteparin and 75 mg of acetylsalicylic acid.

All the surgical procedures in the recipient and the donor were done through a mid-line laparotomy. The object of the donor surgery was to isolate the uterus together with 10-15mm of the vaginal cuff, a portion of the bladder peritoneum and with long vascular pedicles including the bilateral uterine arteries and veins up to the internal iliac vessels, with special care to isolate them from their firm attachment from the ureters. Then, the graft was gently flushed with HTK preservation solution at 4ºC (Custodiol, Nordmedica AS, Gentofte, Denmark).

The preparation of the recipient to receive the graft included dissection and opening of the vaginal vault and the dissection of the external iliac arteries and veins bilaterally. The uterine graft was then placed in its orthotopic position in the pelvis and their vascular pedicles were anastomosed end-to-side to the external iliac vessels. After adequate blood flow through the uterine arteries and veins was checked, the vaginal cuff of the graft was sutured to the vaginal vault

of the recipient and the uterus was fixed to the pelvis by attaching the uterosacral and round ligaments of the graft to those of the recipients. In addition to that, the bladder peritoneum kept in the graft was overlapped with the recipient's bladder fundus.

8.6.4 Immunosuppression

The recipients received standard immunosuppression protocol for kidney transplantation used at Sahlgrenska Transplantation Institute. Induction was achieved with a single bolus of methylprednisolone prior to uterine reperfusion (500 mg; Solu-Medrol®, Pfizer) and Thymoglobulin ® (i v, 2,5 mg/kg bw; Genzyme) or ATG (i v, 5 mg/kg bw; ATG-Fresenius®; Fresenius Medical Care Sverige AB,) just prior to UTx and at a second occasion 12h later.

Postoperative immunosuppression included tacrolimus, (Prograf®/Advagraf®, Astellas) with the aim to keep trough levels of 10-15 ng/ml during the first 2 months, followed by 5-10 ng/ml from the second month and onwards, Mycophenolate mofetil (MMF, Cellcept®, Roche,) was given orally as a single dose preoperatively (1 g). Starting from postoperative day 1, MMF, was administered twice daily aiming for MMF-area under the curve (MMF-AUC) trough levels of 40-60 mg×h/L. MMF was discontinued after six months, well before any embryo transfer, because of its described teratogenic effects. The aim was then to treat the patients with solely tacrolimus or in cases of repeated rejection episodes during the initial 6 months to give continuous azathioprine instead of MMF. Oral prednisolone (5 mg/day) was also administered during the first 5 days post-operatively (Prednisolon, Pfizer, New York, NY, USA).

8.6.5 Follow-up

The recipients were monitored twice a week during the first month, once a week during the second and third months and thereafter the monitoring was

spaced out, depending on decisions on individual patients, according to their record of rejection episodes. Follow-up assessment included physical examination, gynecological examination, ultrasound examination of the graft (2D and Doppler), cervical sampling for cultures and biopsies and blood samples to monitor immunosuppressive drug levels, hemoglobin levels, white blood cell counts, and tests of kidney as well as liver function.

9 RESULTS AND DISCUSSION

9.1 Paper 1- Assessment of the effect of calcineurin inhibitors on implantation

9.1.1 RESULTS

Clinical outcomes: Pregnancy rate was reduced in animals receiving cyclosporine-A (CYA) compared to those receiving tacrolimus or NaCl (CYA10: 58.3%, TAC02: 100%, TAC05: 80%, Control: 80%), although differences were only significant when compared to the TAC02 group (p=0.03). Implantation rate/animal was also significantly lower in the CYA group compared to all of the other groups (CYA10: 32.1%[0%-73.2%]; Control: 57.1%[32.1%-92.9%]; TAC02: 71.4%[64.3%-92.9%]); TAC05: 78.6 [37.5%-94.6%]; p<0.001). Pregnancy and implantation rates are shown in Figure 8.

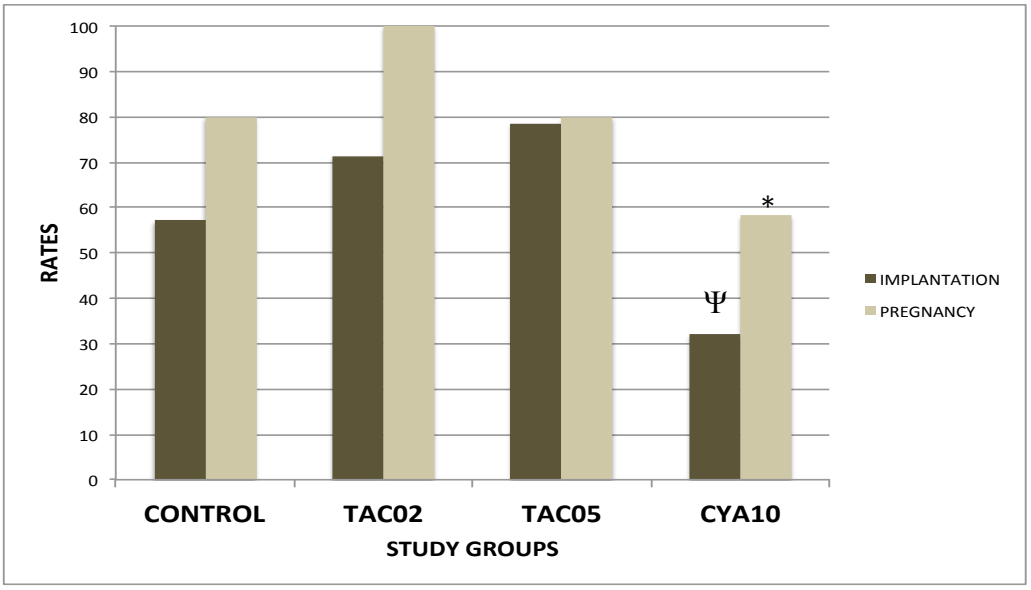


Figure 8. Pregnancy and implantation rates in animals receiving NaCl 0.9% (Control), two different doses of tacrolimus (TAC02: 0.2mg/kg/day; TAC05: 0.5mg/kg/day) or cyclosporine-A 10 mg/kg/day (CYA10). Each animal was transferred 14 donated embryos from a breeding colony. Ψ: p<0.001; *: p=0.03 for comparisons between CYA10 and the other groups.

The sizes of the fetuses were similar between groups, with all pups having crown-rump lengths between 6-8 mm.

Uterine Natural Killer-uNK-cells (DBA+) and activated lymphocytes (CD25+) characterization: Densities of DBA+ cells in both placenta and decidua of pregnant animals were similar between groups. All groups showed surface areas with positive staining varying between 3% and 6% in placenta samples and 16% and 28% in decidua samples. DBA expression in non-pregnant animals was almost absent and restricted to some cells in the subendometrial stroma. Uteri from animals treated with cyclosporine-A showed lower densities of CD25+ cells (0.38% CYA10) than the other experimental groups (0.84% Control, 1.35% TAC0.2 and 1.15% TAC0.5, $p<0.05$).

Expression of markers of implantation and placentation: The studied genes included markers of vascularization and vascular proliferation (CD31, VEGF-A), cell adhesion (ICAM-1), presence of uNKs (NKG2D), molecules that modulate immune responses in the utero-placental junction and drive the implantation and placentation processes such as the non-inflammatory-Th2-like cytokines interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13 and leukemia inhibitory factor (LIF), and the inflammatory-Th1-like cytokines (tumor necrosis factor (TNF)-alpha and TNF-beta). There were no differences regarding the expression of any of these genes in either decidua samples or placental samples.

9.1.2 COMMENTS

This is the first systematic study evaluating the effect of different CINs and providing the accurate calculation of implantation rates, since the exact numbers of embryos transferred were known. In previous studies, exposure to cyclosporine-A at higher doses (30mg/kg/day) was associated with a lower pregnancy rate and decreased implantation rate [102]. An essential limitation of that study was that the pregnancies were secondary to natural mating, and thus the accurate implantation/pregnancy rate could not be determined given that the

number of embryos reaching the uterine cavity was unknown. It is also important to highlight that CINs can affect the ovulation rate (see results from paper #2#), so the natural mating model is not appropriate for studying variables such as implantation.

The main findings of this study were that cyclosporine-A used within a therapeutic range can adversely affect implantation and therefore diminish pregnancy rate. This effect was not observed when the animals were exposed to tacrolimus. Although both are CINs, this differential effect could be explained by the fact that they interact with calcineurin through the intermediation of different molecules (FKBP-12 for tacrolimus, cyclophilin-A for cyclosporine) (Setkowicz *et al.*, 2004). This causes/may cause different conformational changes in the calcineurin subunits, leading to different downstream effects in the intracellular signaling pathways (Setkowicz *et al.*, 2004).

Regarding fetal size, fetal growth retardation is a well-known side effect in pregnancies from immunosuppressed mothers (McKay and Josephson, 2006), and it has also been evidenced in experimental models after term pregnancies (Groth *et al.*, 2010). Nevertheless, in our study, no differences between intervention groups were seen. This difference may be explained by the fact that fetal weight was studied on pregnancy day +12, indicating that fetal growth restriction related to CYA is a late-pregnancy event or that the dose used in our study is too low to cause such effect.

When analyzing uNK markers, markers of vascularization, cell adhesion, or shift in the TH1/TH2 profile, we did not find any differences between groups. Considered together, these data suggest that CINs have no effect on these key players of implantation. On the other hand, CINs have been shown to modulate the activity of NKs from other tissue sources (Lin and Kuo, 2008; Morteau *et al.*, 2010; Howell *et al.*, 2013; Neudoerfl *et al.*, 2013), and it has also been shown that the calcineurin-Nuclear factor of the activated T-cells (CaN-NFAT) pathway is required to achieve the typical Th1 to Th2 shift that has been

classically described in successful pregnancies (Yamashita *et al.*, 2000). CINs impair Th2 cell development more intensively than that of Th1 cells (Yamashita *et al.*, 2000), resulting in a promotion of the Th1 pro-inflammatory profile. These apparently conflicting results between our experiment and previously reported data can be explained by the fact that implantation is a complex and highly organized biological process, in which numerous molecular mechanisms interact in a synchronized time-and-site-specific manner to enable the embryo to properly interact with the decidualized maternal surface (Guzeloglu-Kayisli, 2011). In our experiment we were evaluating one specific time point (pregnancy day +12), and it is possible that even if we could not identify any difference in molecular mechanisms mediating the effect of cyclosporine-A at that time-point, it would have existed earlier in pregnancy. This could be one underlying mechanism helping to explain the decreased implantation rate. Therefore, based on the results of the present experiment, a new study is currently ongoing using similar methods but with samples obtained on pseudopregnancy day +3. The analysis of these data will provide further information enabling us to better characterize the effect of CINs during the implantation window.

9.2 Paper 2-Assessment of the effect of calcineurin inhibitors on ovulation

9.2.1 RESULTS

In these experiments, rats exposed to therapeutic doses of cyclosporine-A showed a decreased ovulation rate compared to control and tacrolimus-treated rats, showing lower median numbers of ovulated oocytes in the Fallopian tubes (CYA10 9 [0-22], TAC05 21 [8-41], Control 22 [6-39], p=0.03). There were no differences between the control group and tacrolimus-treated animals. The use of CINs did not modify the different white blood cell subpopulations in

peripheral blood, nor did they modify the amount of neutrophils (measured indirectly by ELANE expression), T-lymphocytes (CD4+ and CD8+) or macrophages (CD163+). On the other hand, both cyclosporine-A and tacrolimus were found to downregulate MPO expression in the ovary. The anti-proteolytic activity during early post-ovulatory period, measured through RUNX2 and TIMP3, was not significantly different between groups.

9.2.2 COMMENTS

There are conflicting results in the literature regarding the potential effects of transplantation/immunosuppression on ovarian function. While an improvement in fertility parameters, usually associated with normalization of the hypothalamic-pituitary-ovarian axis function, is experienced after transplantation of solid organs [226], ovarian dysfunction in solid organ-transplanted patients has also been described, with mid-luteal phase defects associated with low progesterone levels [227]. Despite these clinical observations, to our knowledge there are no experimental studies on the effects of immunosuppressive drugs on ovarian function.

The biologically central process of ovulation has been compared to a local inflammatory reaction, since a number of inflammatory mediators, as well as some subsets of leukocytes, are essential for carrying out the tissue remodeling of the extracellular matrix, blood flow changes [228], and changes within the cumulus cells [229]. The intraovarian biochemical changes of ovulation are time- and site-specific, and to a large extent involve various types of molecules with antagonistic effects to achieve controlled tissue remodeling. Examples of these molecules are metalloproteinases secreted by resident white blood cells in the ovary, and inhibitors of metalloproteinases secreted by theca cells, granulosa and fibroblasts [230]. Like any other inflammatory process, immunosuppressive drugs that modify the function of the different leukocyte subpopulations could potentially regulate ovulation. Secretion of

metalloproteinase inhibitors from ovarian tissue cells could also be regulated by immunosuppressants due to the ubiquity of the molecular pathways disrupted by these drugs. Therefore, the rationale of this experiment was to assess if CINs can influence ovulation, since ovulation is an inflammation-like process.

Our main finding was that cyclosporine-A, but not tacrolimus, decreases the ovulation rate when administered for at least two estrous cycles. These differential effects on ovulation by these two types of CINs were unexpected, because the main mechanism of both is the specific inhibition of calcineurin, with the most common and serious secondary effect being the cessation of interleukin-2 release from T-lymphocytes. However, in our previous experiment (see results from paper #1#), we already demonstrated this dichotomy. Moreover, as previously discussed in paper #1#, the differential effects of cyclosporine A and tacrolimus have previously been described in other organs and systems [231].

The reduction in ovulation rate in the CyA-group does not appear to be mediated by any major change in leukocyte infiltration patterns as histological examination of the post-ovulatory ovaries did not find any difference in terms of numbers and distribution of neutrophils, macrophages or lymphocytes in the three experimental groups. Similarly, peripheral leukocyte populations did not vary between groups. These two facts indicate that CINs do not affect vascular permeability and recruitment of immune cells to the ovary. Although most of the evidence available in the literature suggests that CINs may affect the chemotaxis and activation of neutrophils and macrophages [232-235], there are also studies suggesting the absence of such effect of CINs [236]. These differences between studies are likely caused by the different doses and route of administration of CINs, the target organ, or the methodology employed.

We also found decreased expression of MPO in CIN-treated animals, indicating that although there is no difference in the number of neutrophils (measured by ELANE), their capacity to produce oxygen-reactive species is diminished. This

effect is consistent with the data from transplanted patients in whom the production of free radical species is decreased by the effect of immunosuppressive drugs [237].

Regarding ovulation markers, we did not find any significant difference between groups, which is in contrast to our clinical observations of the reduced number of ovulated oocytes in the CYA group. An explanation of this finding is that signaling pathways involved in TIMP3 regulation are independent of calcineurin regulation [238]. Therefore, the detrimental effect on the ovulation rate observed in the group of animals treated with cyclosporine-A seems to be independent of the expression of metalloproteinase inhibitors like TIMP3 in ovarian tissue.

It must be highlighted that this study was designed to assess the effect of the main variable—the ovulation rate. Given the complexity of the process of ovulation, this experiment allowed us to only partially study the mechanisms behind such effect. Based on the results, one can rule out that the decrease in ovulation rate caused by calcineurin is due to alterations of leukocytes subpopulation infiltrations. Moreover, it is unlikely that any imbalance in the production of metalloproteinases inhibitors is a factor behind the results. It may well be that other factors, such as changes in production of metalloproteinases [239], abnormal vascularization [240] or follicle contractility [241] are underlying causes of this effect.

9.3 Papers 3 and 4-Development of an experimental model of allogeneic uterus transplantation allowing for pregnancy

9.3.1 Development of the surgical technique (syngeneic)

9.3.1.1 RESULTS

A total of 27 UTx procedures were performed and 19 animals were included in the study, resulting in a surgical success rate of 70%. The causes for exclusion

of 8 UTx animals were: death due to bleeding within the first three post-operative days (n=3), unsatisfactory post-operative recovery (n=1) and pathological findings (graft thrombosis (n=1), intra-abdominal adhesions (n=1), and constricted uterus-uterus anastomosis (n=2) during the check-up surgery. In the included animals, the median graft cold ischemia duration was 120 min (range: 60-210 min) and the duration of the recipient surgery was 215 min (range: 170-270 min). In the Sham group, the duration of surgery was between 35 and 40 minutes and surgical success was 100%.

Breeding performance of the two groups is shown in Table 3. The UTx rats showed a significantly higher number of resorbed pregnancies and a significantly lower number of delivered litters. In 4 out of 7 UTx rats parturition was initiated but could not proceed to delivery. There were no differences between groups regarding the birth weight and weight of the pups, nor in their growth-trajectory (follow-up until post-natal day 60).

Table 3. Breeding performance after UTx and Sham surgery.

	Utx	Sham	Sig.
Animals	19	19	n.s.
Mated (first cycle)	17	16	n.s.
Pregnant animals	11	12	n.s.
Successfully delivered litters	1	8	p<0.01
Total number of pups or fetuses	32	45	
Pups/pregnancy*	3 (2-6)	3.5 (3-7)	n.s.
Number of resorptions	6	0	p<0.01

Numbers express absolute values (ranges)

9.3.1.2 COMMENTS

In the present study the success rate of this orthotopic surgical model was similar to that of previous heterotopic murine models [89-91]. Pregnancy rates and litter sizes were similar between groups. Nevertheless, under the

assumption that each uterine horn carries half the litter, the median number of pups in the present model was lower than that reported by other authors in normal animals [242]. This may result from the transplantation procedure itself, but might also indicate that insult caused by sham surgery affects the fertility potential of the uterus. The increased number of resorbed pregnancies in the UTx group may indicate that conditions supporting fetal survival after UTx are sub-optimal. When the paper was written, it was not clear whether this was caused by altered blood flow, interrupted lymph drainage or denervation, or if it was simply an effect of the small sample size. Later experiments (see paper #5#) did not find this increase of intrauterine death, suggesting that the differences were likely due to the sample size of the experiment.

One of the main findings was the difference in delivery rates. When the pups were examined at euthanasia no differences were noted regarding the fetal size, suggesting that all of them reached full-term and that there was a cause that motivated arrested parturition. There are two potential explanations for such arrest: The lack of innervation of the uterus, since nerve reflexes influence parturition in rodents [243], and the presence of scar tissue in the anastomosis of the vaginal vault of the recipient along with the vaginal cuff of the graft which could have caused a lack of dilatation of the birth canal. Regardless of the actual cause, the main conclusion of this experiment was that caesarean section should be the preferred way of delivery in future experiments.

9.3.2 Fertility experiments in an allogeneic setting

9.3.2.1 RESULTS

Pregnancy rates (defined as number of pregnant females/total number of females) were not significantly different between groups (UTx-Tac-group: 5/8 (62.5%), Sham-Tac-group: 3/5 (60%), Sham-nonTac-group 5/6 (83.3%)). It should be noted that in the UTx group, all pregnancy sites were present in the uterine graft and none were seen in the small native remnant of the uterine

horn. There were no differences between groups in the median [ranges] numbers of fetuses per pregnant rat: 1 [0-3] in the UTx-Tac-group, 1 [1-5] in the Sham-Tac-group and 3 [1-4] in the Sham-nonTac-group. Resorbed pregnancies were seen in 4 out of 5 pregnant rats in the UTx-Tac-group (median 1 resorbed pregnancy [0-3]) and in 1 (1 resorbed) out of 4 and 1 (1 resorbed) out of 5 in the Sham-nonTac-group and Sham-Tac-group, respectively. None of the animals showed macroscopic signs of thrombosis and/or rejection. Uterine morphology (gross/light microscopy) was similar in the uteri of all groups with no signs of inflammation or necrosis in any group.

Tacrolimus levels (median [ranges]) at the end of the experiment were within the same range in the UTx-Tac-group (11.4 ng/ml [4.6-18]) as in the Sham-Tac-group (9.4 ng/ml [5.7-25]).

9.3.2.2 COMMENTS

This experiment proved for the first time that pregnancy after allogeneic uterus transplantation was possible. Pregnancy rates were very close to the 70% pregnancy rate observed in the syngeneic orthotopic rat model (see paper #3#)[92], and were also similar in the number of resorbed pregnancies. As in the previous study, the occurrence of resorbed pregnancies, along with a pregnancy rate far from 100%, in all groups may suggest implantation defects after uterine surgery.

The tacrolimus levels in the UTx-Tac and Sham-Tac groups were close to the upper limit of the therapeutic range. In the liver transplantation program at our hospital, the tacrolimus target levels are half of those achieved in this study (5 ng/ml).

This experiment was designed to make progress toward the development of a allogeneic orthotopic UTx model, and the endpoint was to evaluate the presence of implantation sites and the feasibility of pregnancy. Since caesarean

sections were scheduled on pregnancy day 17, there is no data on the outcome of the offspring for obvious reasons.

9.4 Paper 5-Evaluation of the perinatal outcome and growth trajectory of the offspring issued from mothers transplanted from an allogeneic uterus

9.4.1 RESULTS

The surgical success rate was 71% (10/14) in the UTx group and 100% (10/10) in the Sham and Sham-Tac groups. The causes for exclusions in the UTx group were: one intra-abdominal hemorrhage secondary to leakage from the anastomosis site on post-op day 1; one animal died due to anesthetic complications while replacing a mini-osmotic pump on post-op day 28; one rat presented a necrotic uterus during second-look laparotomy on post-op day 15; and one animal was excluded because of extensive, left-sided hydronephrosis which was found on post-op day 15.

Pregnancy rates were higher in the control groups with sham surgery (70% in Sham group; 80% Sham+TAC group) than in the UTx+TAC group (50%,), although these differences did not reach significance. There were no differences between groups regarding number of living pups or neonatal deaths (Table 4). Litter sizes of the BC+TAC (13[0-14]) group were significantly larger, although also with a greater range, compared to the other experimental groups.

No differences were observed between groups regarding the macroscopic appearance of the uteri. Microscopic examination of the uteri of the non-pregnant rats showed similar endometrial and myometrial patterns in all experimental groups, except for one animal in the Sham+TAC group that showed evident fibrosis in all uterine compartments. Thrombi were present in both arterial and venous vessels in this animal. Variations of different phases of

the estrus cycle were seen in uteri from non-pregnant animals, suggesting that these animals were cycling normally. Evaluation of uteri and placentae of pregnant animals revealed no major disruptions of placental structures in any of the three experimental groups, although the vascular density in both the subendometial location and the spongiotrophoblast was lower in the UTx+TAC group compared to the Sham group and the Sham+TAC group (Figure 9).

Table 4. Fertility and perinatal outcomes after allogeneic UTx in the rat model

	UTx	SHAM+TAC	SHAM	BC	BC+TAC
MATING (1ST cycle)	9/10	10/10	8/10	10/10	10/10
MATING (2ND cycle)	4/6	5/5	6/6	-	-
PREGNANCY RATE	5/10	8/10	7/10	8/10	8/10
LIVE PUPS					
Mothers	5/5	8/8	7/7	7/8	6/8
Pups	5[1-7]	2.5[1-7]	1[1-5]*	5.5[0-14]	13[0-14]*
STILLBORNS					
Mothers	1/5	3/8	0/7	2/8	2/8
Pups	0[0-1]	0[0-1]	0	0[0-7]	0[0-4]
NEONATAL DEATHS					
Mothers	1/5	2/8	1/7	1/8	1/8
Pups	0[0-2]	0[0-2]	0[0-2]	0[0-1]	0[0-1]

Values are presented as raw data for dichotomic variables and median [range] for continuous variables. For each perinatal outcome, "mothers" represent the number of rats whose offspring presented the event/number of pregnant rats; "pups" represent the median [range] number of pups per litter size presenting the event.
*Sham vs BC+TAC: $p<0.05$

No differences were found between groups when the pattern or expression of endothelin receptors A and B were compared (Figure 10). Tacrolimus levels were similar between the tacrolimus-treated animals during mid-pregnancy (UTx+TAC: 1.88±0.60 ng/ml; Sham+TAC: 1.77±0.49 ng/ml; BC+TAC: 1.92±0.45 ng/ml; n.s.) and at the time of C-section/laparotomy (UTx+TAC: 1.50±0.20 ng/ml; Sham+TAC: 1.63±0.63 ng/ml; BC+TAC: 1.71±0.48 ng/ml; n.s.). Male pups from UTx+TAC mothers had higher birth weight than male offspring from the Sham group (6.2±0.2 g vs 5.3±0.1 g; $p<0.001$), but not compared to Sham+TAC group (5.9±0.6 g). There were no differences between experimental groups regarding the birth weight of female offspring

(UTx+TAC: 5.5±0.6 g; Sham+TAC: 5.3±0.3 g; Sham: 5.1±0.5 g). When compared to animals of the breeding colony, both males and females born from UTx+TAC mothers had similar birth weights (UTx-TAC males: 6.2±0.2 g; UTx-TAC females: 5.5±0.6 g; BC males: 5.8±0.2 g; BC females: 5.2±0.3 g; n.s.).

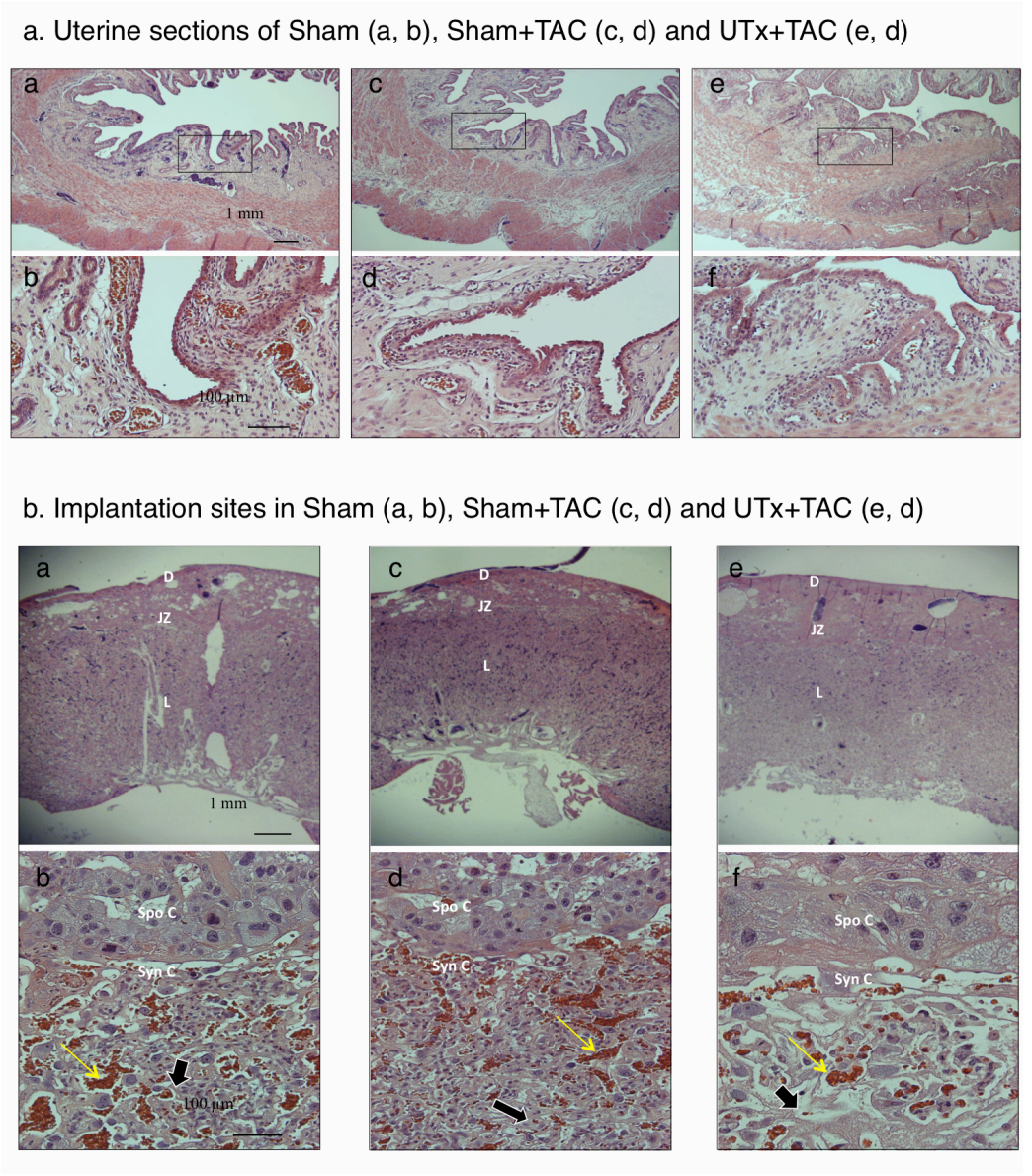


Figure 9. Histological sections of uteri from pregnant rats. H.E. staining. A lower vascular density is seen in the UTx group. Such decreased vascularization pattern is noticed both in non-implantation sites (1a) and implantation sites (1b). L, labyrinth zone; JZ, junctional zone; D, decidual basalis cells; Syn C, syncytiotrophoblast cells; Spo C, spongiotrophoblast cells. The maternal vascular compartment (yellow arrows). The fetal capillary space (black arrows)

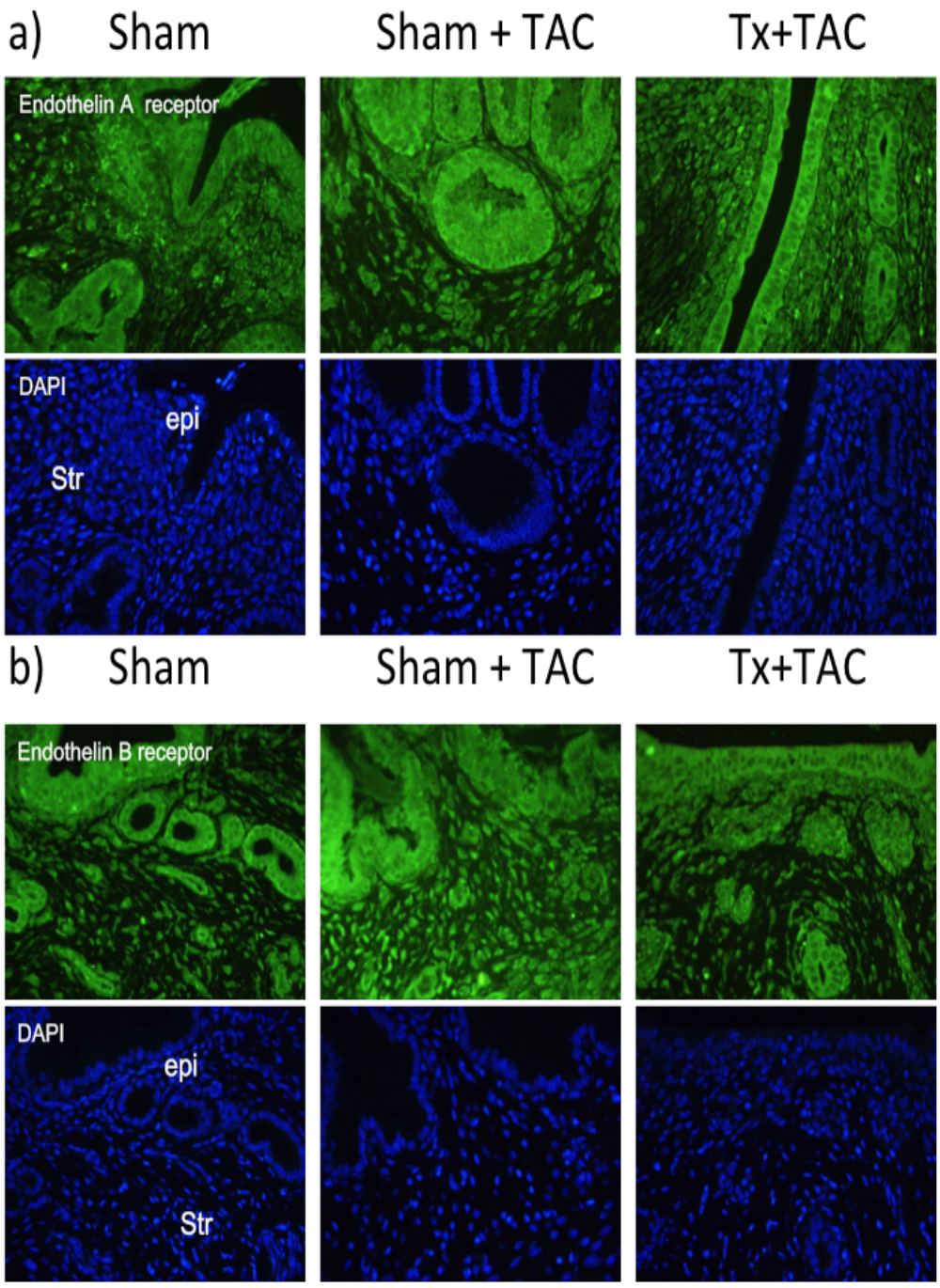


Figure 10. Endothelin receptor expression in uteri from the experimental groups. Endothelin A (a-figures) and B-receptors (b-figures) show a similar distribution pattern between groups when detected by immunofluorescence staining, being expressed in both epithelial (epi) and stromal (Str) compartments.

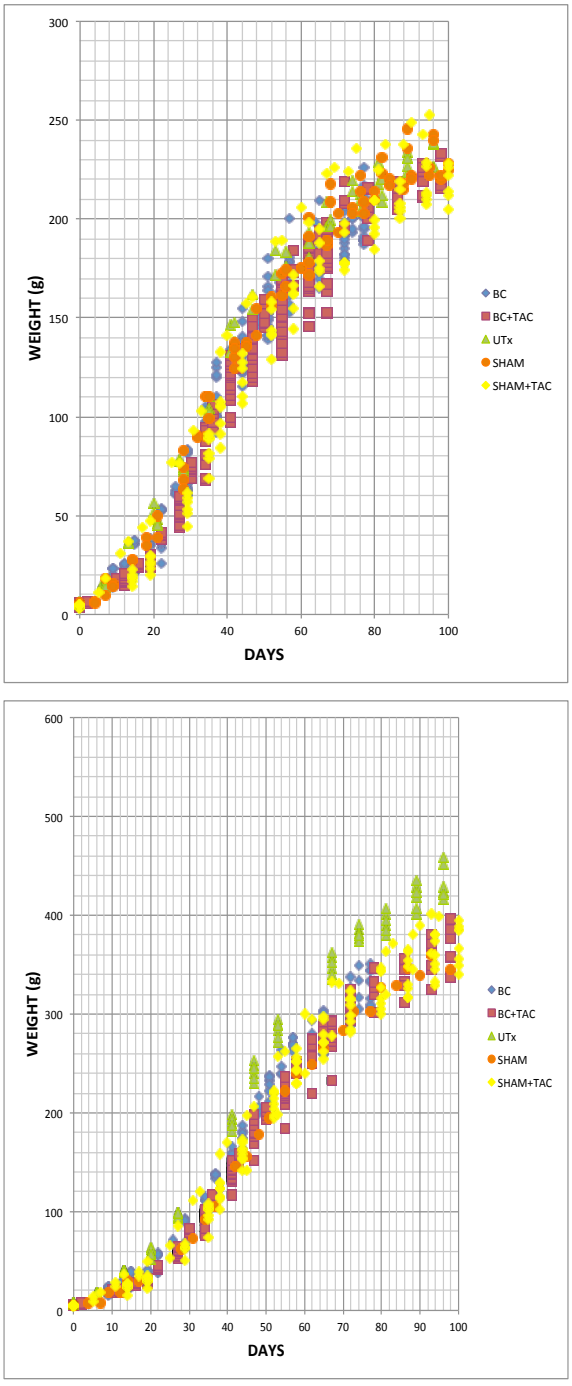


Figure 11. Comparison of weight evolution between pups from the different experimental groups and the two breeding colonies. The "Y" axis presents the weight in grams, the "X" axis presents the age in days. Data are presented for female (upper) and male (bottom) offspring.

The proportion of low birth weight (LBW) pups did not differ significantly between experimental groups (UTx+TAC: 10.5%; Sham: 7.7%; Sham+TAC: 11.3%), nor with the BC group (10% by definition).

Growth trajectories until post-natal week 16 are shown in Figure 11. No weight differences were seen at any time point between female offspring from the different groups. The male offspring from the UTx+TAC groups showed a trend toward higher weight compared to the other experimental groups and to the external breeding colony controls. This difference was significant from postnatal week 10 (Figure 11 bottom) but not at earlier time points.

9.4.2 COMMENTS

The present study evaluates for the first time postnatal outcomes in an allogeneic UTx setting. The

main finding is that the birth weight of offspring is not affected. However, vascular density of the uterus was affected, although the placental weights were unchanged. Moreover, the postnatal growth of male pups from the UTx+TAC group was slightly accelerated after postnatal week 10.

The fertility rate in the UTx+TAC group of the present study is comparable to that of our previous study in an allogeneic rat UTx setting [98]. Animals in all three experimental groups of the present study had smaller litter sizes compared with animals of the breeding colony. A plausible explanation for this is the 50% reduction of potentially implantable endometrium, since all animals of the experimental groups had undergone surgical excision of one uterine horn. The animals in the breeding colony groups (BC and BC+TAC groups) underwent no intervention and had normal bicornuate uteri.

Overall, our data concerning pregnancy rates suggest that the implantation potential of a transplanted uterus is not impaired relative to a native one. This effect was not observed in our previous experiments using an allogeneic UTx model (paper #4#). An explanation for this could be the lower doses of tacrolimus used. However, it could also be an effect of the small sample size of the experiments, since in the syngeneic orthotopic model (paper #3#), in which immunosuppression was not used, the number of resorbed pregnancies was increased. Another fact supporting the hypothesis of a preserved implantation/placentation potential is that offspring born from UTx+TAC mothers did not show lower birth weights or a higher rate of LBW than offspring from the Sham group or the breeding colonies. This, would suggest that the blood flow entering the graft is enough to support a normal pregnancy. Experimental data in several animal species indicate a direct relationship between uterine blood flow and size of offspring. In a model with experimentally lowered uterine blood flow during pregnancy in the rat, the reduced utero-placental perfusion affected birth weight of the offspring,

regardless of whether the reduction began before (day 12) or after (day 14) the initiation of the physiological fetal growth increase [244].

In an allogeneic setting, the effect of immunosuppression to avoid organ rejection is an additional non-physiological factor that has to be taken into account. In this experiment, tacrolimus doses were lower than in our previous experiments using the rat allogeneic model (0.2mg/kg/day vs 0.5 mg/kg/day), so they were tacrolimus blood levels (1.88±0.60 ng/ml vs 11.4 ng/ml [4.6-18]).

The histological analysis revealed that microvessel densities were decreased both in the uterus and the placenta of the UTx+TAC group. Importantly, this was not seen in the Sham+TAC group, which would indicate that it is solely an effect of the surgical tissue trauma, denervation and altered vascular supply/drainage after UTx. Despite this change in microvascular density, fetal growth was not affected. This could be explained by the fact that the complex and not fully understood mechanisms controlling utero-placental blood flow are multifaceted [245], and that compensatory mechanisms exist which neutralize any possible negative effect on fetal growth by the impaired microvascularization mentioned above.

In the present study we also examined the expression of endothelin and its receptor in the uterine tissue. Endothelin is a potent vasoconstrictive substance released by endothelial cells, which can be increased as much as threefold in utero-placental disorders such as pre-eclampsia and fetal growth restriction [246, 247]. Endothelin receptors in patients with pre-eclampsia and LBW offspring are usually down-regulated in uteroplacental circulation. This probably reflects a mechanism aiming to maintain the maternal blood supply to the fetus [245]. In our study, such down-regulation was not seen in any placental sample from either group, indicating that endothelin-mediated responses in utero-placental circulation are not dysregulated by UTx or immunosuppression.

The perinatal variables of the present study are limited to perinatal mortality, birth weight and weight evolution, and further research is needed to ensure that offspring following allogeneic UTx also develop normally at older ages and that other aspects of the phenotype also remain normal. Such investigations are underway in our research group; the cohort of pups from this study are being followed-up for 24 months, which represents two thirds of the life of a rat, and aspects such as behavior, metabolism, urinary function and fertility have been recorded for phenotype characterization.

9.5 Paper 6-Evaluation of the perinatal outcome of pregnancies mothered by transplanted patients

9.5.1 RESULTS

A total of 2511202 births were reported in the general population during the time period and 195 births were from 130 mothers after transplantation. The majority of pregnancies in the transplanted group occurred among renal transplant patients (170 pregnancies, 87%). The other types of organ transplantations among the mothers giving birth were kidney+pancreas (7 pregnancies, 3.6%), kidney+liver (1 pregnancy, 0.5%), liver alone (13 pregnancies, 6.7%) and thoracic organs (4 pregnancies, 2.1%). The vast majority (80.5%) of the 195 deliveries among the transplanted patients occurred during the last two decades (after 1990).

The demographic characteristics of all pregnancies are shown in Table 5. Comparisons between pregnancies from the general population and those from transplanted mothers are shown in Tables 6 and 7. Up to a 9-fold increased of risk of preeclampsia, preterm delivery and instrumental deliveries and caesarean section deliveries were observed in pregnancies from transplanted patients. Perinatal outcomes of the babies of transplanted mothers showed a 3-fold increase of risk of being small for gestational age, having low birth weight,

intrauterine death and perinatal mortality. It has to be highlighted that no increase in the rate of major malformation was seen.

In the sub-analysis of the type of cesarean section (emergency or elective) with recorded data from the time periods 1990-1999 and 2000-2009, the proportion of elective, but not of emergency, cesarean section increased both in the transplanted group and in the general population (transplanted: 45.7% vs 60.9%, $p<0.001$; non-transplanted: 37.5% vs 45.4%, $p<0.001$).

The results of the comparison of pregnancy outcomes within transplanted patients (pregnancies before vs. pregnancies after transplantation) are shown in Tables 8 and 9. In transplanted patients, 1192 transplanted mothers gave birth to 2260 newborns before transplantation. After controlling for confounding factors, no increase in adverse perinatal outcome was found when comparing pregnancies before transplantation with pregnancies after transplantation. To avoid mixing independent and dependent observations, perinatal outcomes were further compared in a pair-wise manner within the same patient giving birth after and before transplantation (Table 10). This part of analysis only included the most recent birth before transplantation and the first birth after transplantation in patients having more than one birth prior to or after transplantation. In this analysis births after transplantation showed a lower incidence of all adverse perinatal outcomes, although no statistically significant differences were evident, except for cesarean section, which was more prevalent after transplantation (OR: 3.2, 95%CI: 1.0-10.1).

When the subgroup of the renal transplant patients was analyzed, similar proportions for preeclampsia (20.6%) and preterm delivery (34.1%) were found as compared to those of the whole transplant population. The proportions of preterm delivery after renal transplantation increased significantly over time, but the proportions of preeclampsia in this renal transplant subgroup remained constant.

Table 5. Demographic characteristics of the general population and pregnancies after solid organ transplantation, Norway 1969-2009

	General population	After transplantation	
Fathers			
Age group			
<19	31782 (1.3)	1 (0.5)	p<0.001
20-24	380835 (15.3)	12 (6.5)	
25-29	780008 (31.4)	45 (24.2)	
30-34	709725 (28.6)	47 (25.3)	
35-39	378431 (15.2)	46 (24.7)	
40-44	141177 (5.7)	25 (13.4)	
45-49	44245 (1.8)	4 (2.2)	
>50	17388 (0.7)	6 (3.2)	
Total	2483591 (100.0)	186 (100.0)	
Mothers			
Age group			
=<19	143372 (5.7)	2 (1.0)	p<0.001
20-24	649775 (25.9)	27 (13.0)	
25-29	857129 (34.1)	58 (29.7)	
30-34	589846 (23.5)	63 (32.3)	
35-39	228408 (9.1)	43 (22.1)	
>=40	42724 (1.7)	2 (1.0)	
Total	2511154 (100.0)	195 (100.0)	
Parity			
0	1038409 (41.4)	105 (53.8)	p<0.001
1	873136 (34.8)	72 (36.9)	
2	409303 (16.3)	17 (8.7)	
3	126986 (5.1)	1 (0.5)	
>=4	63368 (2.5)	0 (0.0)	
Total	2511202 (100.0)	195 (100.0)	
Multiple pregnancies			
Births from twin pregnancies	66836 (2.7)	8 (4.7)	n.s.
Total	2511202 (100.0)	195 (100.0)	

Table 6. Complications during pregnancy and delivery when comparing pregnancies after transplant in mothers with pregnancies in the general population, Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mothers and fathers age, parity and plurality.

	195 births after transplant n (%)	2511202 births in the general population n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Preeclampsia	42 (21.6)	77457 (3.1)	8.7 (6.2-12.2)	6.5 (5.0-9.1)
Preterm delivery (<37 weeks)	66 (34.0)	154027 (6.1)	7.9 (5.8-10.6)	7.1 (5.3-9.6)
Preterm delivery (<34 weeks)	28 (14.4)	51575 (2.1)	8.0 (5.4-12.0)	7.5 (5.0-11.1)
Induction of labor	46 (23.7)	387823 (15.4)	1.7 (1.2-2.4)	1.6 (1.2-2.2)
Operative vaginal delivery	10 (5.2)	170490 (6.8)	1.3 (0.7-2.5)	0.5 (0.2-0.9)
Caesarean section	121 (62.4)	257660 (10.3)	15.0 (10.8-19.4)	9.7 (7.2-13.0)
Apgar<7 at 5 min	2 (1.1)	26779 (1.5)	0.7 (0.2-2.9)	0.6 (0.2-2.6)

Table 7. Perinatal complications and malformations when pregnancies after transplant were compared to pregnancies in the general population, Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mother's age and parity. Low birth weight was also adjusted for gestational age.

	195 births after transplant n (%)	2511202 births in the general population n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Small for Gestational Age (<10 percentile)*	42 (22.7)	269944 (11.3)	2.3 (1.6-3.2)	2.5 (1.7-3.5)
Large for Gestational Age (>90 percentile)*	4 (2.2)	227807 (9.6)	0.2 (0.1-0.6)	0.21 (0.1-0.6)
Low Birth Weight	57 (29.4)	131130 (5.2)	7.5 (5.5-10.3)	2.7 (1.7-4.1)
Stillbirth	5 (2.6)	26081 (1.0)	2.5 (1.0-6.1)	2.9 (1.2-7.0)
Perinatal mortality	7 (3.6)	36554 (1.5)	2.5 (1.2-5.4)	3.2 (1.5-6.7)
Major malformations	8 (4.1)	52359 (2.1)	2.0 (0.99-4.1)	1.6 (0.8-3.3)

*Only 185 transplanted patients and 1798146 non-transplanted patients were considered in the analysis of this variable

Table 8. Complications during pregnancy and delivery when comparing pregnancies after and before transplantation, Norway 1967-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for confounders as given.

	2260 births before transplant n (%)	195 births after transplant n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Preeclampsia	244 (10.8)	42 (21.6)	2.3 (1.6-3.3)	0.97 (0.63-1.49)
Prelabor rupture of membranes	52 (2.3)	12 (6.2)	2.8 (1.5-5.3)	1.47 (0.67-3.20)
Bleeding during pregnancy	127 (5.6)	32 (16.5)	3.3 (2.2-5.0)	1.27 (0.77-2.10)
Preterm delivery (<37 weeks)	318 (14.1)	66 (34.0)	3.1 (2.3-4.3)	1.32 (0.91-1.94)
Induction of labor	489 (21.6)	46 (23.7)	1.1 (0.8-1.6)	0.73 (0.49-1.07)
Operative vaginal delivery	115 (5.1)	10 (5.2)	1.0 (0.5-2.0)	0.57 (0.27-1.21)
Caesarean section	342 (15.1)	121 (62.4)	9.3 (6.8-12.7)	2.81 (1.95-4.03)

All OR are adjusted for year of birth, mother's age and parity.

Table 9. Perinatal complications and malformations when mothers were transplanted, before and after transplantation, Norway 1967-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for confounders as given.

	2260 births before transplant n (%)	195 births after transplant n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Small for Gestational Age (<10 percentile)*	430 (20.4)	42 (22.7)	1.1 (0.8-1.6)	1.16 (0.76-1.76)
Large for Gestational Age (>90 percentile)*	175 (8.3)	4 (2.2)	0.2 (0.1-0.7)	0.26 (0.09-0.74)
Low Birth Weight	325 (14.4)	57 (29.4)	2.5 (1.8-3.5)	1.73 (0.45-1.20)
Apgar <7 at 5 min*	40 (4.1)	2 (1.1)	0.3 (0.1-1.1)	0.12 (0.03-0.53)
Stillbirth	75 (3.3)	5 (2.6)	0.8 (0.3-1.9)	0.53 (0.19-1.47)
Perinatal mortality	106 (4.7)	7 (3.6)	0.8 (0.4-1.7)	0.61 (0.26-1.44)
Infant death	52 (2.3)	2 (1.0)	0.4 (0.1-1.8)	0.37 (0.08-1.71)
Major malformations	57 (2.5)	8 (4.1)	1.7 (0.8-3.5)	0.82 (0.34-1.95)

All OR are adjusted for year of birth, mother's age and parity. Low birth weight is also adjusted for gestational age.

Table 10. Complications during pregnancy and delivery and perinatal outcomes when comparing pregnancies before and after transplantation in the same patient. Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mothers and fathers age, parity and plurality. Low birth weight was also adjusted for gestational age.

	26 births after transplant n (%)	26 births before transplant n (%)	Crude OR (95% CI)
Preeclampsia	5 (19.2)	7 (26.9)	0.6 (0.2-2.4)
Preterm delivery (<37 weeks)	10 (38.5)	10 (38.5)	1 (0.3-3.1)
Induction of labor	5 (19.2)	11 (42.3)	0.3 (0.1-1.1)
Operative vaginal delivery	1 (3.8)	3 (11.5)	0.3 (0.0-3.2)
Caesarean section	19 (73.1)	12 (46.2)	3.2 (1.0-10.1)
Small for Gestational Age (<10 percentile)	6 (23.1)	8 (30.8)	0.6 (0.2-2.2)
Large for Gestational Age (>90 percentile)	0	0	
Low Birth Weight	11 (42.3)	13 (50)	0.7 (0.2-2.2)
Stillbirth	1 (3.8)	5 (19.2)	0.2 (0.0-1.6)
Perinatal mortality	1 (3.8)	5 (19.2)	0.2 (0.0-1.6)
Major malformations	1 (3.8)	1 (3.8)	1 (0.1-16.9)

Only the last birth before transplantation and the first birth after transplantation were considered in the analysis. Only crude OR are displayed, given the wide range of the CI for AOR.

9.5.2 COMMENTS

One particular strong point of this study is its uniqueness, of studying a complete nationwide population-based study and thereby free of underreporting and selection bias as compared to other voluntary registries of pregnancy in transplanted patients, like the registries supported by transplant societies mainly covering Europe and the USA [197].

The presence of an increased rate of pregnancy complications, as seen in the present study, confirms earlier findings of a generally increased risk of several pregnancy-associated problems, when compared to the general population of pregnant women. These complications may affect both the transplanted mother and her infant. Our finding of 21.6% preeclampsia among the transplanted females is somewhat lower than previously reported in large studies of data from voluntary registries such as the UK Transplant Pregnancy Registry (36% preeclampsia rate) and the US National Transplant Pregnancy Registry (29% preeclampsia rate) [196]. On the other hand, the results are similar to those from a Swedish population-based study of births after maternal organ transplantation [199] (22% preeclampsia rate). Interestingly, our data shows that the proportion of preeclampsia does not increase over time and it could thus not explain the observed increase seen in preterm delivery.

A striking difference in our results compared to previous reports from voluntary registry data [196, 197, 248-252] is the much lower rate of preterm delivery that we report. The overall rate of preterm delivery in our material of transplanted mothers was 34% and a sub-analysis of time trends, revealed an increase over time. Previous studies with data mostly accumulated from voluntary transplant pregnancy registries from Europe, UK and the US have indicated rates of preterm delivery among organ transplanted mothers of around 50%, but with some variations depending on type of organ transplantation. The

very high odds ratio of preterm delivery (7.1) in our material, despite the relatively lower rate of preterm birth, is due to a very low rate of preterm delivery in the general Norwegian population (6.1 %), which is about half of what is reported in the general US population [253]. This difference in overall preterm delivery proportion may also be partly dependent on differences in routines for delivery of high-risk pregnancies.

Differences may exist in preterm rate depending on the specific type of transplanted organ of the mother. Earlier studies have indicated an increased rate of preterm delivery among renal transplant patients as compared to liver or heart transplant patients.[197]. However, the proportion of preterm delivery in our data was similar when examining renal transplant patients alone as compared to the entire group of transplanted women.

When analyzing births before and after transplantation, we could not detect any significant increase in adverse perinatal outcomes after adjustment for confounding factors. These data are in line with those published by Kallen and coworkers, [199] suggesting that the morbidity status of the patients could be the underlying cause of adverse outcomes. Moreover, when comparing births within the same patient before and after transplantation, we found a lower rate of preeclampsia, preterm birth, growth restriction problems and perinatal mortality, indicating that the transplant may have a protective effect on both fetus and mother. This could be explained by a better general health of the women after transplantation, which of course may be partly counterbalanced by possible negative effects of immunosuppression. It bears mentioning that, in our series, the incidence of adverse pregnancy events was lower than in that of the only previous population study, which compared pregnancies before and after transplantation [199] in a Swedish population. This was true, even if we assessed the risk of four additional obstetric outcomes: induction of labour, instrumental delivery, caesarean section delivery and low APGAR score.

The risk of being subjected to cesarean section in the transplanted patients was high. Interestingly, the rate of planned cesarean section increased over time in transplanted women. This increase was also seen in the general population. A plausible explanation for the increased planned cesarean section rate among the transplanted patients is that this subgroup would follow the general trend, although the cesarean section is not necessarily medically motivated.

The main limitations of this study are related to three different facts:

-The high proportion of renal transplanted patients (87%): a predisposing factor for preeclampsia in renal transplant patients is that a large proportion of these patients experience both hypertension and proteinuria after transplantation, which may make them more prone to develop preeclampsia. Moreover, these patients with a single functioning kidney would have a 2-3 fold increased risk of preeclampsia, which is indicated by studies of preeclampsia rates among kidney donors, before and after kidney donation. These facts could limit the external validity of the results to patients transplanted from other type of organs.

-The low prevalence of congenital malformations, which also makes it difficult to draw conclusions about risks related to any specific malformations. However, there was no increased risk for congenital malformations overall, which is reassuring. It has also to be pointed out that diseases potentially related to immunosuppression and manifested later in life, such as autoimmune disease [254], were not included in our study.

-The low number of patients and events when comparing births before and after transplantation within the same patient did not allow us to control for confounding factors when the regression analysis with propensity score was applied. As each patient was her own control and births were successive and close in time, we do believe that potential confounders would play little or no role in modifying risk estimates. Even if the number were low, it has to be

highlighted that our study included more than twice the number of births after transplantation as compared to the study of Bengt Källén and coworkers [199].

9.6 Papers 7,8,9-description of the first live birth after allogeneic uterus transplantation in the human

9.6.1 RESULTS

9.6.1.1 Patients

Thirty patients were initially offered to participate in the UTx trial. Ten of them met the inclusion/exclusion criteria and nine of them were finally included in the study. One of the recipients was excluded due to the presence of pelvic bilateral kidneys. The basal characteristics of the donors and the recipients are shown in Table 11.

Table 11. Donors and recipients' characteristics

	Recipients	Donors
Age (years)	31.5 (3.9)	53.0 (7.0)
BMI (kg/m2)	22.4 (1.5)	25.6 (4.2)
Previous smoking n(%)	3 (33)	4 (44)
Previous abdominal laparotomy n(%)	2 (22)	3 (33)
Previous abdominal laparoscopy n(%)	6 (67)	-
Single kidney (orthotopic) n(%)	3 (33)	-
Single kidney (pelvic) n(%)	1 (11)	-
Cause of absent uterus, n (%)		
MRKH	8 (89)	-
Cervical cancer	1 (11)	-
Type of vagina, n (%)		
Normal	1 (11)	9 (100)
Self-dilated	3 (33)	-
Therapeutically-dilated	1 (11)	-
Skin	4 (44)	-
Pregnancies	-	3.3 (1.3)
Live births	-	3.0 (0.9)
Vaginal deliveries n(%)	-	25 (93)
Cesarean section n(%)	-	2 (7)
Premenopausal state n(%)	-	4 (44)
Menopausal < 5 years n(%)	-	2 (22)
Menopausal >5 years n(%)	-	3 (33)

Values are means and standard deviations (between brackets) or absolute numbers and percentages.

9.6.1.2 Surgical outcomes

A summary of the surgical outcomes is presented in Table 12.

Table 12. Surgical, anesthesiologic, and hospitalization parameters.

	Recipients	Donors
Duration of anesthesia (hours and minutes)	9h4min (3h14min)	12h13min (60min)
Duration of surgery (hours and minutes)	4h46min (30min)	11h37min (1h5min)
Duration of back-table (hours and minutes)	1h18min (23min)	
Duration of vein anastomosis (minutes)	36 (11)	
Duration of arterial anastomosis (minutes)	30 (6.9)	
Duration of warm ischemia (minutes)	83 (9)	
Duration of cold ischemia (minutes)	78 (23)	
Duration of hospitalization (days)	6.7 (1.6)	6 (0)
Complications (Clavien-Dindo classification)		
Grade I	-	-
Grade II	4 (44)	-
Grade III	2 (22)	1 (11)
Grade IV	-	-
Grade V	-	-

Values are means and standard deviations (between brackets) or absolute numbers and percentages.

Donor surgeries lasted 11 h 37 min and recipient surgeries 4h 46 min in average. The longest cold ischemic period was 2 h and the longest warm ischemic period was 1 h 38 min. One of the donors had a major complication: a ureterovaginal fistula that had to be surgically corrected. After ureteric reimplantation, around 3 months after hysterectomy, she has not had any medical problems. Two of the recipients under went hysterectomy during the postoperative period. One early graft loss on was postoperative day 3 and this was secondary to thrombosis. The patient was heterozygote for the Leiden mutation and this may have been a contributing factor. The histopathology report confirmed extensive thrombosis formation in both uterine arteries and veins. Another patient underwent hysterectomy on postoperative day 105. She acquired an intrauterine infection by *enterococcus faecalis* around 1.5 months after UTx. Despite repeated attempts with iv antibiotic treatment and surgical drainage, she developed an intrauterine abscess. Around 3 months after UTx she showed initial signs of septicemia and it was decided to perform a

hysterectomy. The histopathology report showed patchy necrosis of the uterine wall.

9.6.1.3 Summary of the follow-up

The other 7 patients that had their uteri intact resumed regular menstruations within the first two months after transplantation. Endometrial development was normal, reaching median thicknesses of 14mm [11-18] during the mid-luteal phase and normal Doppler indexes of the uterine arteries were recorded in all the patients.

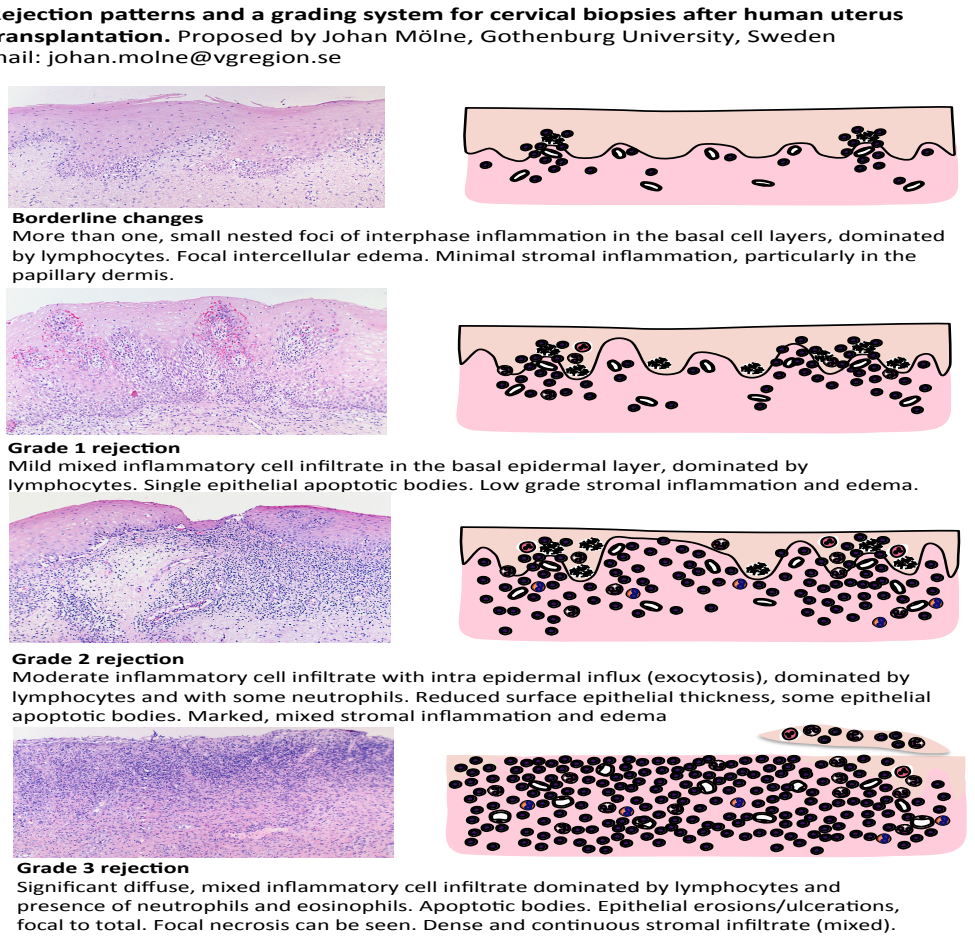


Figure 12. Rejection score developed by Johan Mölne based on our previous experience in the rodent and non-human primate experience.

Rejection episodes occurred in 5 patients, and all of these rejections were subclinical. The diagnosis was made based on the pathological findings of the per-protocol cervical biopsies (mainly neutrophil and lymphocyte infiltration of the epithelial/stromal interfase-Figure 12). All the rejection episodes were successfully treated with corticosteroids (500mg methylprednisolone i.v./day for 3 days and then 10 mg prednisolone/day for 7 days).

One of the patients experienced a cervical intraepithelial neoplasia type 2 (CIN 2), positive for HPV 31. A loop conization was performed with a cone biopsy showing normal epithelium and no signs of remaining CIN.

9.6.1.4 Description of the first pregnancy after allogeneic UTx in the human

One year after transplantation, a 35-year-old patient with a type B MRKH syndrome with just one kidney and uterovaginal agenesis was the first patient to get pregnant and subsequently she would deliver a healthy baby. She received the uterus from a 61-year-old donor not genetically linked to the patient. The donor had two previous vaginal deliveries at term and she had been menopausal for seven years before the transplant. Sequential oral contraceptives were administered for three months before transplantation to test the functionality of the uterus.

During the postoperatative follow-up the patient presented three episodes of rejection that were successfully treated with our standard protocol for acute rejection episodes. Even more, she was the patient undergoing conization due to a CIN2, as described in the previous paragraph.

This patient had 11 embryos from three previous IVF cycles. Ten months after transplantation, MMF was discontinued and azathioprine at 2mg/kg/day was introduced. It has to be pointed out that prednisolone 5m/day was also kept in the patient after her third episode of rejection. Twelve months after UTx the patient got pregnant after her first single embryo transfer (SET) which was of a day 2 embryo in the natural cycle. All maternal and fetal controls during

pregnancy were normal until week 31 except for hemoglobin values in the lower range of the normality from the beginning of the pregnancy, requiring administration of ferric carboxymaltose and darbepoetine alpha. On pregnancy day 31+5 the patient came for the normal pregnancy control but was kept at the obstetric department, since preeclampsia was diagnosed. She had a blood pressure of 180/120 mmHg, proteinuria, low platelet count (96,000/microL) and the CTG of the fetus showed variable decelerations. Therefore a cesarean section was done and a neonate of 1775g (normal weight for date) APGAR 9-9-10 was successfully delivered. The baby was doing fine after birth and did not need respiratory aid (oxygen or positive airway pressure). The inspection of the uterus revealed a normal graft that contracted correctly after the cesarean section and all their vessels were patent.

9.6.2 COMMENTS

The series of nine patients represent the first successful attempt to treat AUFI. The duration of the surgery was about 2-3 times longer than expected based on our previous experience in the non-human primates [73, 142] and the radical hysterectomy model [72]. Careful dissection of the tunnel of the ureter, just beneath the uterine artery, is a consuming step of the surgery, but this step is unavoidable if a living donor is used. After that step the uterine arteries and veins have to be dissected to include parts of the internal iliacs. The difficulty is mostly on the venous sides, since there are lots of venous branches and connections between the uterine veins. These have to be divided and sutured, one by one, since ligations or coagulation, will lead to bleeding after reperfusion of the organ.

The only complication we evidenced in our donors also took place at this anatomic site: one of the patients got a ureterovaginal fistula due to ureteric damage probably related with the dissection of the ureter with use of too much diathermy close to the ureter. Thus, the complication occurred was evident first

10 days after uterus retrieval, which is a typical time for the development of thermal damage of the relatively thick ureteric wall. In the human case published by the group of Fageeh [69], they experienced such a ureteric laceration, but that was evident at surgery and could be repaired immediately. The occurrence of these two complications, highlights the difficulties linked to the surgical step of the dissection of the tunnel of the ureter, which is a component of a live donor UTx but not in a deceased donor situation.

Two of the patients lost their uteri during the postoperatory follow-up. The patient who lost the uterus due to thrombosis during the early post-op was a heterozygous carrier of a protein C deficiency, but this is not a formal contraindication for transplantation. She was under anti-aggregation and anti-thrombotic therapy and it is not clear if a shorter surgery could have minimized the risk of thrombosis. Another contributing factor, may have been that the uterine artery of the 62-year old uterus of this case were the thinnest in our study and consequently the measured blood flow at perfusion was the lowest. The second patient who lost the uterus due to infection was exposed to different potential risk factor that may have influence the lost of the graft: extended anesthesia time of almost 14 hours, vagina-to-vagina anastomosis placed very close to the cervix and of course the immunosuppression protocol.

Despite these adverse effects in these three patients, it has to be kept in mind that this is the first series of a novel type of surgery and further experiences will provide an optimization of the procedure.

The protocol of monitoring of rejection after transplantation was based in our previous experience with baboons [142]: cervical biopsies were easy to take and they correlated well with the clinical findings in this non-human primate species. In this trial all the acute rejections episodes were subclinical, so there could be a that we were overtreating. The fact that all the histological findings normalized after corticoid administration makes overtreatment a very unlikely hypothesis. The normal menstrual pattern, normal endometrial growth and the

normal vascular indexes found during ultrasound examination points toward a normal vascular influx during the non-pregnant state of the graft.

Regarding the fertility outcomes, issues such as the time window between transplantation and embryo transfer, the suitability of IVF prior to transplantation and the fact of doing SET have been discussed before ("ART in uterus transplantation" section). Immunosuppression during pregnancy seemed to work better that before pregnancy, with only one episode of rejection as compared to the three previous episodes before pregnancy. Pregnancy itself can induce a local immunosuppression state and this could be beneficial for the graft. On the other hand physiologic requirements increase during pregnancy and some of the immunosuppression-related side-effect can become apparent, for example the anemia caused by anti-metabolites, the diabetogenic effect of tacrolimus and corticoids and the nephrotoxicity of tacrolimus. Anemia and increased levels of creatinine were evidenced in our pregnant patient and the doses of immunosuppressants had to be tapered (azathioprine was diminished from 2 mg/kg/day to 1.2 mg/kg/day).

The development of preeclampsia during pregnancy may be related to different factors, all of them present in our patient: the more obvious is the fact of being transplanted [197], although after our population-based study, concerns about the real effect of transplantation could be raised; the patient was pregnant after IVF using frozen embryos and the transplanted uterus was beyond the age of sixty. Both pregnancy after IVF [255] and advanced maternal age [256] have been associated to an increased risk of preeclampsia; the last factor that could have had increased the risk of preeclampsia was that the patient only had one kidney: there exist data in the literature suggesting and increased rate of preeclampsia in healthy patients after kidney donation as compared to the double kidney population [257].

The removal of the uterus after birth will allow diminishing the incidence of side effects of immunosuppression, but it was not performed at the time of the

cesarean section in order (1) to let the uterus return to its normal size, which would simplify the hysterectomy procedure, (2) to ensure the health of the delivery baby and (3) to discuss with the patient about the completion of her reproductive wishes.

10 CONCLUDING REMARKS

This thesis has proven for the first time that uterus transplantation is a feasible option to treat AUFI, allowing for live birth in humans. This opens a new door for women suffering from this type of infertility to become genetic mothers, especially in socio-political contexts where surrogacy is not allowed.

Additionally, it provided reassuring data from population studies suggesting that transplantation and immunosuppression have moderate to no effect on the apparition of adverse perinatal outcomes when other confounding factors linked to the basal status of the mother are controlled. Complementing this is data from the murine model developed in this thesis, which showed normal evolution of the offspring born from mothers with a transplanted uterus.

Finally, this thesis has proven that the calcineurin inhibitor cyclosporine-A, but not tacrolimus, decreases ovulation and implantation rates, which can be taken into account when planning the future fertility projects of transplanted women.

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13 LIST OF PUBLICATIONS

This is thesis is based in the following publications. Reprints were made with permission from the publishers.

I. Effects of immunosuppressive calcineurin inhibitors on implantation rate in the mouse.
C. Díaz-García, R. Akouri, M. Hellström, S. Herraiz, S. Lozano, M. Brännström

Submitted to Human Reproduction

II. Differential effects of the immunosuppressive calcineurin inhibitors cyclosporine-A and tacrolimus on ovulation in a murine model
César Díaz-García, Farnosh Zakerkish, Loida Pamplona, Edurne Novella, Mats Brännström

In Manuscript

III. Pregnancy after syngeneic uterus transplantation and spontaneous mating in the rat
Caiza A. Wranning, Shamima N. Akhi, Cesar Diaz-Garcia, and Mats Brännström

Human Reproduction. 2011;26(3):553–8.

IV. First report on fertility after allogeneic uterus transplantation
César Díaz-García, Shamima N. Akhi, Ann Wallin, Antonio Pellicer, Mats Brännström

Acta Obstet Gynecol Scand. 2010;89(11):1491-4.

V. Pregnancy after allogeneic uterus transplantation in the rat: perinatal outcome and growth trajectory.
Díaz-García C, Johannesson L, Shao R, Bilig H, Brännström M.

Fertil Steril. 2014;102(6):1545-52.

VI. Pregnancy outcome after maternal solid organ transplantation: nationwide population-based cohort study over four decades

C. Diaz-Garcia, M. Brännström, A.V. Reisæter, A. Foss, T. Leivestad, O. Geiran, N-H. Morken

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VII. First clinical uterus transplantation trial: a six-month report.

Brännström M, Johannesson L, Dahm-Kähler P, Enskog A, Mölne J, Kvarnström N, Diaz-Garcia C, Hanafy A, Lundmark C, Marcickiewicz J, Gäbel M, Groth K, Akouri R, Eklind S, Holgersson J, Tzakis A, Olausson M.

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VIII. Uterus transplantation trial: 1-year outcome

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IX. Livebirth after uterus transplantation

Brännström M, Johannesson L, Bokström H, Kvarnström N, Mölne J, Dahm-Kähler P, Enskog A, Milenkovic M, Ekberg J, Diaz-Garcia C, Gäbel M, Hanafy A, Hagberg H, Olausson M, Nilsson L.

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Effects of immunosuppressive calcineurin inhibitors on implantation rate in the mouse.

Running title: Calcineurin inhibitors and implantation

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Extended Abstract

Study question: Do cyclosporine-A and tacrolimus impair pregnancy/implantation rates when used at therapeutic doses for immunosuppression?

Summary answer: Cyclosporine-A but not tacrolimus impairs pregnancy/implantation rate.

What is known already: Molecular mechanisms controlling implantation and placental development are susceptible to modulation by the calcineurin inhibitors that are used after virtually all types of allogeneic of organ/composite tissue transplantation. There exist some data suggesting that cyclosporine-A could negatively affect implantation after spontaneous mating in the rat but with uncertainties because of lack of data concerning numbers of implantable embryos. There exist no information of the effect of the more modern calcineurin inhibitor tacrolimus on implantation.

Study design, size, duration: Experimental design. Random allocation to intervention.

Participants/materials, setting, methods: Adult female B6CBAF1 mice (n=43) were allocated to one of the following immunosuppressant treatments: 1)tacrolimus 0.5mg/kg/day (TAC05; n=10); 2)tacrolimus 0.2mg/kg/day (TAC02; n=11); 3)cyclosporine-A 10mg/kg/day (CYA10; n=12); 4)normal saline 0.9% (Control; n=10). Fifteen days after commencement of immunosuppression, the mice underwent transfer of a specified number of blastocysts. On pregnancy day +12, animals from all the experimental groups underwent caesarean section in order to assess implantation rate, fetal viability and tissue sampling for uterine natural killers (uNK)/placentation markers/cytokines characterization by immunohistochemistry and RT-PCR.

Main results and the role of chance: Pregnancy rate in the CYA10 group was lower than the control and TAC groups, although differences were only significant when compared to the TAC02 group (CYA10: 58.3% vs TAC02: 100%; p=0.03). Implantation rate was also significantly lower in the CYA group as compared to all the other groups (CYA10: 32.1%[0%-73.2%]; Control: 57.1%[32.1%-92.9%]; TAC02: 71.4%[64.3%-92.9%]); TAC05: 78.6 [37.5%-94.6%];

p<0.001). No differences in numbers of uNK or in cytokine profiles were evidenced between groups.

Limitations, reasons for caution: Immune responses and molecular mechanisms mediating the effect of calcineurin inhibitors may differ from mice to humans, which could limit the extrapolation of this study to a clinical set-up.

Wider implications of the findings: The differential effects observed between tacrolimus and cyclosporine-A could imply that tacrolimus should be the preferred calcineurin inhibitor in organ-transplanted women during the reproductive ages as the preferred calcineurin drug in transplanted women with fertility wish.

Study funding/competing interest(s): Swedish Research Council funded the study.

Key words: cyclosporine, implantation, pregnancy, tacrolimus, uterus

Introduction

Solid organ transplantation has become a common procedure nowadays, with more than 114000 patients being transplanted with solid organs in 2012 (Global observatory on donation and transplantation. (Organización Nacional de Transplantes and World Health Organization. Organ and transplantation activities 2012. Available in http://issuu.com/o-n-t/docs/2012ad). Up to 38.4% of the transplanted patients are women and among them 17.8% are still in reproductive age (18-34 years), according to the Organ Procurement and Transplantation Network (OPTN) data as of August 31, 2014.

The effects of organ transplantation and the accompanying immunosuppressant regiments on subsequent fertility are poorly understood. It is well known that there commonly is a recovery of fertility after organ transplantation, since the women go from an end-stage disease with its major systemic influences to having a near to normal organ function (Douglas *et al.*, 2007). Higher rates of adverse perinatal outcomes including preterm delivery, low birth weight and preeclampsia have been described after transplantation in studies using data from large registries of pregnancies after organ transplantation (McKay and Josephson, 2006).

To what extent the effects of transplantation on fertility and perinatal outcomes are due to the underlying disease being the cause of transplantation or to the use of immunosuppressive medication is a question that has not been fully investigated.

Implantation is a tremendously complex and highly organized biological process, in which numerous molecular mechanisms interact in a synchronized time-and-site-specific manner to enable the embryo to proper interaction with the decidualized, maternal surface (Guzeloglu-Kayisli, 2011). Implantation defects can interfere with further events like placentation, that in turn may be related to obstetrical complications of placental origin such as intrauterine growth restriction and preeclampsia (Kim *et al.*, 2003).

Several of the molecular mechanisms controlling implantation and placental development may be susceptible to negative modulation by immunosuppressants used to prevent rejection of a transplanted organ. Especially important in this context are the calcineurin inhibitors (CIN) tacrolimus and cyclosporine-A, with either of them being the main agent in all types of organ transplantation. The CINs bind to the calcium-dependent serine-threonine-protein-phosphatase, calcineurin (CaN) together with the so-called tacrolimus binding proteins (FK506BPs or FKBPs) or immunophilins. The immunosuppressive effect of CIN is mainly achieved through the interaction of CaN+FKBP12+tacrolimus or CaN+immunophilin-A+cyclosporine-A (Huai *et al.*, 2002). Such interactions block the phosphatase activity of CaN and prevent dephosphorylation of the transcription factor NFAT, inhibiting the expression of IL-2, which in turn leads to decreased proliferation and activation of T-lymphocytes. However, CINs could also negatively affect other biological processes by binding other types of immunophilins that are involved in modulating the transcriptional activity of different steroidal receptors, for example those driving implantation and placentation, like PR-receptors and also by modulating the uterine natural killers (uNK) repertoire and activity (Lin and Kuo, 2008; Morteau *et al.*, 2010; Howell *et al.*, 2013; Neudoerfl *et al.*, 2013).

In a previous report (Groth *et al.*, 2010) from our group, we demonstrated that cyclosporine-A treatment of rats may result in a lowered pregnancy rate after natural mating and also that cyclosporine in-utero exposure of female offspring was associated with a significant reduction in fetal weight. Nevertheless, there were two main limitations in that study and animal model used. Firstly, natural mating was used and thus the true numbers of fertilized eggs and subsequent number of implanted embryos in each animal were unknown. Ovulation rates and fertilization rates can also show differences between litters and strains. Secondly, there were no comparison between the two major CINs, cyclosporine-A and tacrolimus. Although the major mechanism of action to prevent rejection is similar, the effects of these two CINs differ in many aspects such as

effects on renal function, vascular function and metabolic profile (Setkowicz *et al.*, 2004). Thus, their effects on the physiological events of the reproductive tract tissues could also be different.

The aim of the present study was to elucidate if different doses of the most widely used CINs, cyclosporine-A and tacrolimus modify the implantation rate in an embryo transfer murine model in which the exact amount of embryos to be implanted is known. We examined fetal numbers and weight during mid pregnancy in order to investigate if there exist any effect of the CINs on the fetal size, and if any, to investigate if they take place during early or late stages of embryogenesis/fetal development and the parallel placentation process. We also examined molecular mechanisms involved in placentation that could be potentially affected by the use of CINs, focusing on gene expression of markers of vascularization and vascular proliferation (CD31, VEGF-A), cell adhesion (ICAM-1), presence of uNKs (NKG2D), and other molecules that modulate immune responses in the utero-placental junction, driving the implantation and placentation processes. These are the non-inflammatory, Th2-like cytokines interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13 and leukemia inhibitory factor (LIF) and the inflammatory, Th1-like cytokines (tumor necrosis factor (TNF)-alpha and TNF-beta).

Materials and methods

Study design

Adult female B6CBAF1 mice (n=43) were randomly allocated to receive daily doses of one of the following immunosuppressant treatments: 1) tacrolimus 0.5mg/kg/day (TAC05; n=10); 2) tacrolimus 0.2mg/kg/day (TAC02; n=11); 3) cyclosporine-A 10mg/kg/day (CYA10; n=12); 4) normal saline 0.9% (Control; n=10). Pseudo-pregnancy was induced in the experimental groups, 10 days after starting the immunosuppression and five days after pseudo-pregnancy induction, the mice underwent embryo transfer of blastocysts derived from donors of the same strain (14 blastocysts -7 blastocysts/horn- were transferred per mouse). On pregnancy day 12 (7 days after

embryo transfer), animals from all the experimental groups underwent cesarean sections in order to assess implantation rate, fetal viability and tissue sampling for NK/placentation markers characterization by immunohistochemistry and RT-PCR.

Ethical approval

The study was approved by the local animal ethics committee in Göteborg, and was carried out according to the principles and procedures outlined in the *NIH Guide for Use of Laboratory Animals*.

Animals

Females (6–8 weeks of age), proven fertility males (2-to 4 months-old) and vasectomized males (see below) of F1 hybrids of C57BL/6xCBA/ca mice (B6CBAF1) were used in this experiment. All B6CBAF1 males and females were obtained from Charles River (Chatillon-sur-Chalaronne, France). The animals were housed in controlled conditions (21–23°C, relative humidity of 50–60%, illumination between 07:00 and 19:00 h) and had free access to water and pelleted food.

Half of the B6CBAF1-males were vasectomized to be used for induction of pseudopregnancy in blastocyst recipient females. Briefly, the animal was anaesthetized with 2% isoflurane (Baxter; Kista, Sweden), and through a low midline incision the bladder was reached and located and the bilateral vas deferens were identified. A 4-0 silk suture was used to place two firm knots around the vas deferens, about 4-5 mm apart. Before cutting out a section of vas deferens, bipolar diathermy was applied over the vas deferens in between the two knots. After repeating the procedure on the contralateral side the midline incision was closed by interrupted sutures of the abdominal fascia, followed by skin-closure with three metallic clips. Two weeks after surgery, the effectiveness of the sterilization was checked by placing the males in a cage with 4-week-old B6CBAF1 female mice that had been superovulated by an i.p. injection of 5 IU pregnant mare's serum gonadotrophin (Sigma-Aldrich Co., St Louis, MO, USA) followed 46 to 48 h later by an i.p. injection of 5 IU hCG (Sigma-Aldrich Co., St Louis, MO, USA). The females were examined for vaginal copulation plugs the next morning, and 46 h after hCG treatment the oviducts were

flushed with G-MOPS plus medium (Vitrolife AB, Göteborg, Sweden) using a 30-gauge needle to examine any possible the presence of oocyte/embryos. In all cases only unfertilized stage oocytes were found under the close examination under a dissection microscope.

Pharmaceuticals

The antibiotic cefuroxime (Zinacef, Glaxco Smith Kline, Uxbridge, UK) was given once immediately after any surgical procedure, s.c. at 40 mg/kg.

Cyclosporine-A and tacrolimus were delivered by the means of subcutaneous miniosmotic pumps (model 2004, Alzet Osmotic Pumps, Cupertino, CA, USA). Each pump was loaded the day before insertion with a tacrolimus solution in 0.9% NaCl or a cyclosporine-A solution in 90% propylene glycol (Fluka, Buchs, Switzerland), adjusted for the weight of the animal, in order to release 0.2 or 0.5 mg/kg/day of tacrolimus or 10mg/kg/day of cyclosporine. This pump was subsequently inserted s.c. in the back of the neck under isoflurane anesthesia. The skin incision was closed with 2-3 metallic clips and the animal was placed under a heating lamp until fully awake.

Blastocyst production

B6CBAF1 females and B6CBAF1 males of proven fertility were used to obtain blastocysts: females were introduced to males in a 2:1 fashion on the day of pro-oestrus. The day after mating, all females were screened for the presence of seminal vaginal plug. The mated females underwent cervical dislocation at 12:00 on day 4 and the uteri were flushed with G-MOPS plus medium (Vitrolife, Sweden). The obtained medium was examined under a Nomarski microscope to evaluate morphology of blastocysts. Only morphologically normal blastocysts were used for embryo transfer.

Mating procedure and embryo transfer

One week after insertion of the miniosmotic pump, the 8 to 10−week−old B6CBAF1 female mice were mated with vasectomized males in order to induce pseudo-pregnancy and

subsequently embryo transfer, with embryos from normally mated and synchronized female mice. The day after mating, all females were screened for the presence of seminal vaginal plug. Only mated females were included in the study.

The embryo recipient females received blastocysts at 12:00 on day 4 of pseudopregnancy, by transmyometrial approach as previously described (Hogan *et al.*, 1994). The blastocysts were held in Gamete□20 medium (Vitrolife, Göteborg, Sweden) for 20–30 min at 37°C and under 5% CO_2. Under isoflurane anaesthesia, two dorsolateral incisions (10 mm) were made on both sides of the midline. The ovary, oviduct and proximal end of the uterus were externalized on each side, and seven blastocysts were transferred to each uterine horn, using a 30□gauge needle initially to penetrate the uterine wall; this was followed by transfer of blastocysts through a glass transfer pipette. The abdominal wall was closed in two separate layers with 6-0 sutures. All mice were placed under heat until fully awake and subsequently placed with one vasectomized male in the same cage.

Assessment of implantation and tissue sampling

On pregnancy day 12, all the animals were anaesthetized and underwent laparotomy, through a midline incision. After opening of the uterine horns, the number of viable fetuses was recorded. The number of resorbing implantation sites was also recorded. The size of all pups was measured. Fetuses and placentas were carefully dissected out and uterine and placental biopsies were taken and placed in 4% formaldehyde and RNA-Later for histology and gene-expression analysis.

Tacrolimus and cyclosporine-A levels

Samples of whole blood (250μl) were drawn from the aorta at the day 12 laparotomy. Tacrolimus levels were measured in whole blood by an automated chemiluminescent immunoassay (CMIA) developed for use on the ARCHITECT system (Abbott Scandinavia AB, Sweden). The interassay coefficient of variation varies between 1.16 % and 1.88 %. Cyclosporine-A levels were

measured by enzyme immunochemistry using a CyA-specific assay (Emit®2000, Dade Behring, Milton Keynes, UK), according to manufacturer's instruction. The inter-assay coefficient of variation was below 9%.

uNK characterization by histochemistry

Immunohistochemistry was performed on 4 μm thick tissue sections after they had been deparafinized in xylene, rinsed in ethanol and brought to water through a series of decreasing concentrations of ethanol. Sections were washed with 1% H2O2 in phosphate buffered saline (PBS), and then blocked for 1h with 10% horse serum + 10% bovine serum albumin (BSA) in PBS with 0.1% Tween 20. Slides were subsequently probed with 50 μg/ml biotinylated DBA (Sigma-Aldrich, St. Louis, MO, USA) in 2% horse serum + 2% BSA in PBS for 1h at room temperature. Detection was performed using the streptavidin-horseradish peroxidase complex. Sections were counter-stained with hematoxylin. Ten sections per uterus were assessed by two blinded observers (SL, SHR), using a Leica microscope (LEICA DM4000B) and a calibrated eye-grid at (10X) magnification. Quantification was performed by the same observers (SL, SH) based on high quality images (2048 x 1536 pixels) captured by the Image ProPlus 6.3 software (Media Cybernetics, Rockville, MD, USA).

Gene expression analysis of implantation and placentation markers

Total RNA was extracted from samples stored in RNA later (Sigma-Aldrich, St. Louis, MO, USA) of all experimental groups using the 'Trizol method' according to the protocol recommended by the manufacturer (Life Technologies Inc., Gaithersburg, MD, USA). cDNA retrotranscription was taken out with the MMLV enzyme contained in the Advantage™ RTfor-PCR kit (Clontech, Saint-Germain-en-Laye, France) using the oligo (dT) 18 as primer.

Specific Taqman probes were assayed by quantitative Real-Time PCR to assess the relative expression of the genes coding for a number of proteins (interleukin (IL)-4, IL-10, IL-5, IL-6, IL-13, CD31, vascular endothelial growth factor (VEGF)-A, the natural killer lectin-like receptor

subfamily member 1 (NKG2D), tumor necrosis factor (TNF)-alpha, TNF-beta, intercellular adhesion molecule-1 (ICAM-1), leukemia inhibitory factor (LIF; Table 1). PCR reaction mixtures were prepared with 100 ng of cDNA as a template in the 1xTaqman Gene Expression Master Mix following the manufacturer´s protocol. Then samples were amplified in the 7900HT Fast PCR system (Applied Byosistems, Life Technologies Inc., Gaithersburg, MD, USA). PCR conditions consisted in an initial activation of uracyl-N-glycosylase at 50°C for 2 min., followed by AmpliTaq Gold activation at 95°C for 10 min. Then samples were cycled 40 times by denaturation at 95°C for 15 seconds, and an annealing extension at 60°C for 1 min per cycle.

After controlling the amplification efficiency of the targets genes, the relative expression was obtained by the comparative Ct method (DDCt). The expression of target gene mRNA was normalized with the expression of the Actin-beta reporter gene.

Statistics

Variable values are presented as median and interquartile ranges [p25-p75] between square brackets for continuous variables except for CINs levels, that followed a normal distribution and they were presented as means ± standard deviations. Categorical variables were presented as absolute values and percentages. Continuous variables were compared using the Kruskall-Wallis test and also doing two-by-two comparisons with the control group using the Mann-Whitney's U-test. Tacrolimus levels were compared with the Student's t-test. Categorical variables were compared using the chi-square test and the Fisher exact test when needed. Differences showing a $p<0.05$ were considered as significant.

Results

Fertility outcomes

Data related to pregnancy and implantation rates are displayed in Table 2. Pregnancy rate in the CYA10 group was lower than the control and TAC groups, although differences were only

significant when compared to the TAC02 group (CYA10: 58.3% vs TAC02: 100%; p=0.03). Implantation rate/animal was also significantly lower in the CYA group as compared to all the other groups (CYA10: 32.1%[0%-73.2%]; Control: 57.1%[32.1%-92.9%]; TAC02: 71.4%[64.3%-92.9%]); TAC05: 78.6 [37.5%-94.6%]; p<0.001). All pups had crown-rump lengths between 6-8 mm and no differences were seen between groups (data not shown).

Tacrolimus and cyclosporine-A levels

Concentration of cyclosporine in the CYA10 group was 120.25±42.28 ng/ml. Concentration of tacrolimus in TAC05 group was significantly higher than that of TAC02 (4.14±2.03 ng/ml vs 1.05±0.45 ng/ml; p=0.02)

uNK characterization

Densities of immunostained cells in both placenta and decidua of pregnant animals were similar between groups. All groups showed surface areas with positive staining varying between 3% and 6% in placenta samples and 16% and 28% in decidua samples (Fig 1A-G). Unspecific staining from fetal membranes was also seen in all samples (Fig 1A, C, D). DBA expression in non-pregnant animals was almost absent and restricted to some cells in the subendometrial stroma (Fig 1H). Uteri from the CyA group showed lower densities of CD25+ cells (0.38%) than the other experimental groups (0.84% Control, 1.35% TAC0.2 and 1.15% TAC0.5, p<0.05).

Expression of markers of implantation and placentation

The gene expression of the studied genes did not differ between treatment groups when analyzed in decidua samples nor in placental samples (Table 3).

Discussion

To our knowledge this is the first systematic study in any species evaluating the effect of different CINs on the implantation process and the development of pregnancy and the utero-placental unit. In our previous studies in a natural mating model, CYA exposure before and during

pregnancy inversely correlated with the number of viable implantation sites resulting in live fetuses. Pregnancy rates were also compromised when the animals were exposed to high doses of cyclosporine-A (30mg/kg) (Groth *et al.*, 2010). An essential limitation of that study was that the pregnancies were secondary to natural mating, and thus the accurate implantation/pregnancy rate was not known, since the numbers of embryos reaching the uterine cavity were unknown. In the current study the number of transferred embryos was defined by protocol, herein the implantation rate can be accurately calculated.

Our results confirmed that the implantation rate was significantly decreased in CYA-exposed animals, even at doses of only one third (CYA10-group) of that used in our previous experiments involving natural mating (Groth *et al.* 2010). The CYA blood levels of the animals in the CYA10 group was around 100 ng/ml, which is within the normal therapeutic level in the human.

Contrary to what we anticipated, implantation and pregnancy rates in the TAC-treated animals (TAC02 and TAC05) were not different to that of controls. Furthermore, there was no tendency to a dose-response effect in the TAC-treated animals, since results were equivalent in TAC02 and TAC05 groups. It should be pointed out that the blood levels of the TAC05 group were within the normal therapeutic range in humans.

As mentioned above, the doses chosen in this study are equivalent to those used in the clinical set-up in transplanted patients , and the differences observed between the experimental groups could be explained by the fact that different CINs cause CaN inhibition through the interaction with different molecules (immunophilins). While TAC binds to the FKBP immunophilin family (mainly to FKBP12), CYA binds to cyclophilin-A. The fusion of these immunophilin-immunosuppressant complexes to CaN blocks its phosphatase activity and subsequently inhibits the calcium-dependent dephosphorylation of the transcription factor nuclear factor of activated T cell (NFAT), a factor that controls gene expression in many cell lines, not only immune cells but also endometrial and stromal cells (Abraham *et al.*, 2012). The different effects caused by TAC and CYA can be explained by the fact that FKBP-TAC complexes bind to different

311 aminoacids of CaN as compared to cyclophilin-A-CYA complexes. This phenomenon induces
312 different conformational changes in the catalytic and regulatory subunits of CaN, resulting in
313 different degree of inhibition. The differential effects of CINs have also been described in other
314 organs and systems and they are not exclusive to the reproductive organs (Setkowicz *et al.*, 2004).
315 Fetal growth retardation is a well-known side effect in pregnancies from immunosuppressed
316 mothers (McKay and Josephson, 2006). In the present study we also evaluated this parameter,
317 and at investigation of fetal size on pregnancy day +12, no differences between intervention
318 groups were seen. In our previous experiments involving natural mating female pups born from
319 mother treated with high doses (30mg/kg) of CYA presented lower birth weight than control
320 animals (Groth *et al.*, 2010). This difference may be explained by the fact that fetal weight was
321 studied on pregnancy day +18, indicating that fetal growth restriction related to CYA is a late-
322 pregnancy event or that the dose used in our previous study was higher than of the present study.
323 A uterine unique cell type is the uNK, which are abundant in the uterus and with a suggested
324 major role both during implantation and placentation (Ruocco *et al.*, 2014). The uNKs are
325 recruited into the decidua and placenta early in pregnancy and they peak at mid pregnancy in the
326 mouse, around gestational day +8, and they subsequently decrease in numbers (Paffaro *et al.*,
327 2003). The amount of uNKs present in placenta and decidua did not differ between groups when
328 measured by immunohistochemistry (DBA) and qRT-PCR (NKG2D) and it was comparable to
329 that found in previous reports when assessed in pregnancy day +12 (Paffaro *et al.*, 2003). These
330 facts indicate that if there were any effects of CIN on NK, they did not affect the amount of
331 uNK at that time-point. On the other hand, CINs have also been shown to modulate the activity
332 of NKs from other tissue sources (Lin and Kuo, 2008; Morteau *et al.*, 2010; Howell *et al.*, 2013;
333 Neudoerfl *et al.*, 2013). Uterine NKs produce different INF-γ, which regulate artery remodeling,
334 decidualization and placentation through the induction of VEGF-A expression (Ashkar *et al.*,
335 2000), but in the present study we could not demonstrate any difference regarding the expression
336 of this pro-angiogenic factor. VEGF-A also controls angiogenesis and cell adhesion through the

CIN-NFAT pathways, but again, the endothelial marker CD31 and the adhesion molecule ICAM-1 expression was similar in all the experimental groups.

Another important factor that contributes to the maintenance and progression of pregnancy is the balance between the cytokines produced by T-helper cells in the placenta and the decidua. This balance contributes to the so called immuneprivileged status of the pregnant uterus (Druckmann and Druckmann, 2005). CaN-NFAT pathway is required to achieve the typical Th1 to Th2 shift that has been classically described in successful pregnancies (Yamashita *et al.*, 2000) and CINs impair Th2 cell development more intensively than that of Th1 cells (Yamashita *et al.*, 2000), resulting in a promotion of the Th1 pro-inflammatory profile. Although an explanation of implantation and placentation based on Th1 and Th2 immunologic profiles could be simplistic taking into account the complexity of such processes (Druckmann and Druckmann, 2005; Guzeloglu-Kayisli, 2011; Ruocco *et al.*, 2014), alterations of such clusters of cells/cytokines have been frequently associated with infertility, recurrent miscarriage and placental pathology like preeclampsia or growth retardation (Druckmann and Druckmann, 2005; Guzeloglu-Kayisli, 2011; Ruocco *et al.*, 2014). In our experiment, the expression pattern of pro-inflammatory, Th-1-like cytokines (TNFα and TNFβ) and that of the anti-inflammatory, Th-2-like (IL-4, IL-5, IL-6, IL-10, IL-13 and LIF) did not differ between groups, indicating little or no general effect on the cytokines of the CIN in the placenta/decidua.

In summary, this study shows that cyclosporine-A but not tacrolimus decreases implantation and pregnancy rates in a mouse model when used at doses similar to those of the clinical practice for immunosuppression. This fact indicates that tacrolimus should be used instead of cyclosporine-A as the drug of choice in women with fertility wishes that need calcineurin inhibitors.

One limitation of the present experiments is that outcomes were evaluated on pregnancy day +12 in order to clearly determine implantation sites and fetal size differences. Herein it is possible that even if we could not identify any difference in molecular mechanisms mediating the effect of

cyclosporine-A at that time-point, it would have existed earlier in pregnancy, which could be consistent with the decreased implantation rate. Therefore, further research is needed to investigate these mechanisms during the implantation window. Such investigations are underway in our research group, with a similar experiment evaluating the effect of the CIN cyclosporine-A and tacrolimus on the mouse uterus three days after mating. The analysis of these data will provide further information to characterize the effect of CIN during the implantation window.

Author's role

CDG is responsible of the design of the experiment, surgical procedures, evaluation of the results and writing of the article. REA has participated in surgical procedures, IVF procedures and writing of the article. MH has run and analyzed all the PCR assays. SH and SL have participated in histological evaluation of the samples. MB has participated in the writing of the article and global supervision of the study.

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Conflict of interest

The authors declare that they have no conflict of interest to jeopardize the impartiality of the research reported herein

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Figure Legends:

Figure 1

Uterine sections (2.5x) showing the DBA positive pattern of pregnant animals from Control (A), TAC 0.2 (B), TAC 0.5 (C) and CyA (D) groups. Detail of DBA positive cells found in the myometrial layers from TAC05 sample (E). Placental DBA positive cells within the labyrinth, spongiotrophoblast and Giant cell layers from TAC05 group (F). Reichert´s membrane showing DBA + signal in CyA group (G). Sample from a non-pregnant control showing very low DBA signal (H).

Table 1. References of the probes used for gene expression analysis

Gene	UniGene sequence reference	Reference of the probe (Life technologies)
Actin-beta	Mm.328431	Mm00607939_s1
Interleukin-4	Mm.276360	Mm00445259_m1
Interleukin-5	Mn.4461	Mm00439646_m1
Interleukin-10	Mn.874	Mm00439614_m1
Interleukin-13	Mn.1284	Mm00434204_m1
CD31	Mm.343951	Mm01242584_m1
VEGF-A	Mn.282184	Mm01281449_m1
ICAM-1	Mn.435508	Mm00516023_m1
NKG2D	Mn.8217	Mm00473603_m1
IL-6	Mn.1019	Mm00446190_m1
LIF	Mn.4964	Mm00434762_g1
TNF-alpha	Mn.1293	Mm00443258_m1
TNF-beta	MN.87787	Mm00440228_gH

Table 2. Pregnancy and implantation rates after embryo transfer on recipients under calcineurin inhibitors treatment.

	Control (n=10)	**CYA10 (n=12)**	**TAC02 (n=11)**	**TAC05 (n=10)**
Pregnancy	8/10 (80%)	7/12 (58.3%)[1]	11/11 (100%)	8/10 (80%)
Total number of embryos transferred	140	168	154	140
Total implantation sites	81	62	115	93
Live foetuses/mother	8.0 [1.5-13.0]	4.5 [0-10.0]	10 [7.0-13.0]	10.5 [4.5-13.0]
Resorbed pregnancy/mother	0 [0-0.8]	0	0 [0-3.0]	0 [0-1.0]
Implantation rate	57.1 [32.1-92.9]	32.1 [0-73.2][2]	71.4 [64.3-92.9]	78.6 [37.5-94.6]

[1]:CYA10 vs TAC02: $p<0.03$

[2]:CYA vs Control: $p<0.001$; CYA vs TAC02: $p<0.001$; CYA vs TAC05: $p<0.001$.

Table 3. Gene expression levels in placenta and decidua.

	PLACENTA				DECIDUA			
GENE	**CONTROL**	**TAC02**	**TAC05**	**CYA10**	**CONTROL**	**TAC02**	**TAC05**	**CYA**
CD31	1,12 (0,86)	0,98 (0,47)	0,93 (0,59)	0,86 (0,04)	1,02 (0,20)	0,89 (0,37)	1,06 (0,18)	0,96 (0,19)
VEGF-A	1,03 (0,38)	0,94 (0,17)	0,84 (0,36)	0,98 (0,08)	0,44 (0,29)	0,67 (0,64)	0,61 (0,95)	0,76 (0,19)
ICAM	1,04 (0,63)	1,02 (0,69)	1,03 (0,84)	0,81 (0,28)	1,01 (0,28)	0,87 (0,69)	1,06 (0,44)	0,94 (0,51)
NKG2D	1,26 (0,89)	0,67 (0,47)	1,01 (0,95)	0,71 (0,38)	0,99 (0,31)	0,98 (0,39)	1,06 (0,75)	0,96 (0,29)
IL4	0,65 (0,39)	0,82 (0,19)	0,81 (0,36)	N/A	0,79 (0,85)	1,03 (0,89)	1,07 (0,67)	0,73 (2,24)
IL5	0,89 (0,77)	0,96 (0,23)	0,99 (0,32)	0,92 (0,38)	0,79 (0,57)	0,84 (0,64)	0,87 (0,44)	0,97 (0,77)
IL6	0,74 (1,56)	0,74 (1,18)	0,76 (1,85)	0,81 (0,85)	1,10 (0,53)	0,84 (0,57)	1,03 (0,53)	0,91 (0,44)
IL10	0,85 (1,38)	0,75 (0,58)	0,70 (0,78)	0,82 (0,42)	0,78 (0,74)	1,05 (0,58)	0,92 (0,47)	0,85 (0,63)
IL13	0,44 (0,53)	0,79 (0,27)	0,90 (0,91)	0,25 (1,03)	0,41 (1,20)	0,65 (0,49)	0,75 (0,43)	0,82 (0,75)
LIF	0,86 (1,17)	0,94 (0,25)	0,83 (0,24)	0,79 (0,11)	0,75 (0,85)	0,71 (0,50)	0,70 (0,60)	0,82 (0,44)
TNFa	1,17 (0,66)	0,97 (0,26)	1,01 (0,26)	0,89 (0,20)	0,72 (0,55)	0,86 (0,40)	0,87 (0,58)	0,86 (0,28)
TNFb	0,37 (1,40)	0,33 (0,87)	0,31 (0,71)	0,31 (0,04)	0,72 (1,14)	0,92 (0,92)	0,64 (0,58)	0,70 (0,47)

Data are expressed as medians of the fold change (Interquartile range).

Comparisons were made for each specific gene using the control group as reference group.

No statistically significant differences were found for any comparison.

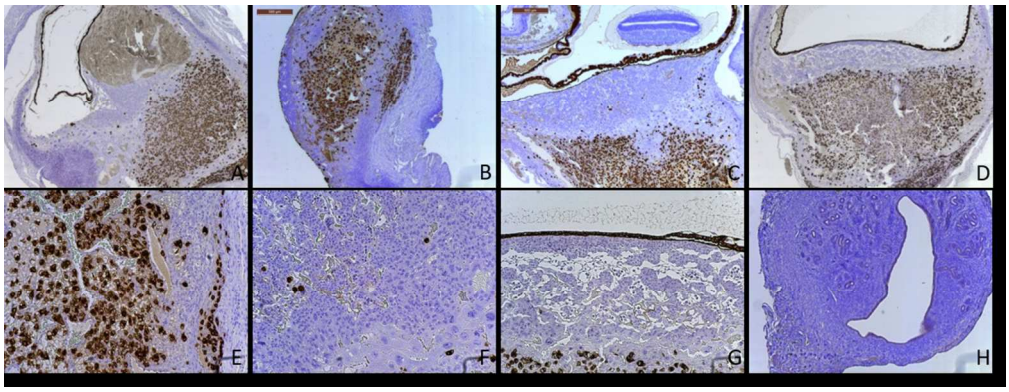

Figure 1
102x39mm (300 x 300 DPI)

Title: Differential effects of the immunosuppressive calcineurin inhibitors cyclosporine-A and tacrolimus on ovulation in a murine model.

Short title: Immunosuppression and ovulation

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Contributions of the authors:

CDG and MB contributed equally to the design of the experiment, analysis of the data and writing of the manuscript. FZ performed writing of manuscript and compilation of

data. The animal work was done by CDG in collaboration with FZ. Immunostaining and related techniques were performed by EN, LP, FZ and CDG.

Abstract

Introduction: Thousands of fertile-aged women around the globe are treated with any of the two calcineurin inhibitors, cyclosporine-A or tacrolimus, to prevent rejection of the transplanted organ that they are carrying. It is unclear whether these drugs affect fertility. Since ovulation is an inflammation-like process with pivotal roles for a number of immune modulators and immune cells it is possible that the calcineurin inhibitors, with broad effects on the immune system, could affect this biologically important process.

Objective: To examine whether therapeutic levels of cyclosporine-A and tacrolimus affect ovulation in the murine gonadotropin-induced ovulation model

Methods:

Results:

Conclusion: This is the first demonstration that cyclosporine negative affects ovulation in murine models and the findings suggest the preferred calcineurin inhibitor for organ transplanted females that hare aiming for pregnancy should be tacrolimus, rather that cyclosporine A.

Key words

cyclosporine, calcineurin, immunosuppression, ovulation, ovary, rat, tacrolimus, transplantation.

Introduction:

The use of allogeneic transplantation of solid organs and composite vascularized tissues in the treatments of life-threatening diseases or to compensate for loss of body function has increased tremendously during the last decades. The key to the success of these treatments and the thereby the increased number of transplantation procedures performed and the expansion with non-life saving organs/tissues, such as the hand, face, intestine and pancreas was the introduction of effective immunosuppression therapy, in the form of the calcineurin inhibitors cyclosporine A in the early 1980s. This was followed a decade later by the introduction of another calcineurin inhibitor, tacrolimus[1].

In parallel with the expansion of the patients groups that are treated with transplantation, the general survival of the patients and general health of the transplanted patients have improved considerably. The medical focus on the transplanted patient has also, apart from graft survival with restored organ function, come to include several issues related to quality of life, with fertility being one important issue. Thus, in year 2006 more than 14000 births had been reported after the first post-transplantation pregnancy, which took place more than 50 years ago[2]. Although the rate of obstetric complications seems to be increased among immunosuppressed women carrying transplants, the risk of fetal malformation is similar to that of the normal population[2].

The effects of transplantation on subsequent fertility are poorly understood. A recovery of fertility after organ transplantation has been widely described in the literature[3], but this has been linked to the considerable improvement of the health status of the female when she is changing from a severely ill patient with end-stage disease into one with restored and normal organ function. There may exist negative effects of the immunosuppressive drugs on the function of the reproductive organs. Animal studies in rodents suggest that the implantation rate is decreased and the miscarriage rate is increased due to exposure to cyclosporine[4,5]. There also exist case reports on affected ovarian function after organ transplantation in women[6].

Ovulation is a complex biochemical process that has a duration of around 36 hours in humans and approximately 12 h in rodent species. During this ovulatory process structural and functional changes occur and this allows the release of a fertilizable oocyte and the transformation of the follicle into a corpus luteum. Several mediator pathways induce changes in the follicle that are necessary for follicular rupture, where breakdown of the extracellular

matrix on the follicular apex [7] and expansion of the extracellular matrix around the cumulus cells (Eppigre) seem to be the most important components for normal ovulation to occur. These processes bear many similarities to an inflammatory reaction, with participation of classical inflammatory mediators such as cytokines, collagenases, stromelysins, gelatinases and prostaglandins as well as pivotal roles for certain leukocyte subsets[8,9]. Several of these inflammatory pathways could potentially be affected by the immunosuppressive effects of calcineurin inihibitors[10].

Recent attempts of human uterus transplantation[11] have further shed the light on the issue of possible effects of immunosuppressant drugs on ovarian function. Since this is the first type of allogeneic transplantation where restored fertility is the goal and with menstruation being the first obvious sign of normal ovarian function is important to ascertain that ovarian function proceeds normally. An indication that ovarian function is disturbed after uterus transplantation is that in the first two cases of attempts to perform human uterus transplantation exogenous estrogen-progestagen therapy were initially given to induce menstrual bleedings. However, in contrast a majority of the patient in the subsequent first clinical uterus transplantation resulting in live birth[12], had regular spontaneous menstruations from 2-3 months after transplantation[13]. To our knowledge there exist no systematic research studies on the effects of immunosuppressant drugs on ovarian function after any type of solid organ transplantation, where kidney transplantation make up around x % of transplantations among women of fertile age. The main type of immunosuppressant in all immunosuppressant protocols is the calcineurin inhibitor, with either cyclosporine-A or the more modern drug tacrolimus, being the drug of choice. In the present study we have for the first time evaluated the effects of the calcineurin inhibitors on the ovulatory process in any species.

Material and methods

Experimental design and animals:

Immature Sprague-Dawley rats (Harlan Nederlands, Horst, Netherlands) were used in this experiment. The animals arrived to our facilities at the age of 16 days and were then housed in controlled conditions (21-23˚C; illumination 0700-1900h) and had access to food ad-libitum. At the age of 21 days, all animals were weighed and then randomly allocated to 3 different groups (15 animals/group): group 1-0.9% NaCl (Control); group 2-cyclosporine-A 10mg/kg/day (CyA) and group 3-tacrolimus 0.5mg/kg/day (TAC05). All animals were synchronized for ovulation at 25 days of age, according our standardized protocol for ovulation studies.[14] Briefly, rats aged 25 days were injected with 10 UI of equine chorionic gonadotrophin (eCG) s.c. at 12:00. Forty-eight hours later, the rats were administered 10 UI of human chorionic gonadotrophin (hCG)(Both purchased from Sigma-Aldrich, Stockholm, Sweden AB, Sweden), with ovulation occurring 10-15 h after hCG administration[15]. Twenty hours after ovulation induction the animals underwent euthanasia, blood samples were taken for white blood cell quantification and measurement of calcineurin inhibitors levels and ovulation rate was assessed. Both ovaries were also taken for histological and molecular studies. The present study was conducted in accordance with Swedish legislations and was approved by the Animals Ethics Committee in Gothenburg.

Calcineurin inhibitors administration:

Miniosmotic pumps (Alzet, Durec Corp, Cupertino, CA, USA) were filled with a solution of tacrolimus (diluted in NaCl-pump model 1007D) or cyclosporine-A (diluted in 1,2-propanediol-pump model 2001) prepared individually for each animal weight. Miniosmotic pumps were filled just with 0.9% NaCl to be used in the control group. Two different models (1007D and 2001) of pumps, with identical external composition, were used since different concentrations of drugs were studied. The pumps were primed in NaCl at 37ºC 12 h prior to its insertion according to the manufacturer's instructions. The first day of intervention (postnatal day 21), a skin incision (5 mm) was made in the back of each animal caudally to the neck, under isofluorane anesthesia. The pump was placed subcutaneously in a parasagital position, and the skin subsequently closed with two polyglactin stiches (Vicryl 4-0, Ethicon, Somerville, NJ, USA).

Calcineurin inhibitors levels:

Samples of whole blood (250µl) were drawn from the aorta at ovulation assessment. Tacrolimus levels were measured in whole blood by an automated chemiluminescent immunoassay (CMIA) developed for use on the ARCHITECT system (Abbott Scandinavia AB, Solna, Sweden). The coefficient of variation between runs for this assay varies between 1.16 % and 1.88 %. Cyclosporin levels were measured by enzyme immunochemistry using a CyA-specific assay (Emit®2000, Dade Behring, Milton Keynes, UK), according to manufacturer's instruction. The inter-assay coefficient of variation was below 9%.

White blood cells subpopulations in peripheral blood

Blood samples were obtained by direct puncture of the aorta during euthanasia. The blood was spread on a glass slide and dried in air. Afterwards it was fixed by immersion in Romanowsky stock solution during 5 minutes and subsequently rinsed in distilled water. Then, the slides were stained for 25 minutes with the diluted Romanowsky solution (1:15 in HEPES buffer pH6.8+DMSO). After staining the samples were rinsed in distilled water and dried in air and cells identified according to their specific characteristics[16].

Assessment of ovulation rate and preservation of ovaries

The ovulation rate was assessed 20 hours after injection of hCG. The oviduct was gently dissected from the ovaries and the uterine horns. The left ovary was kept in RNAlater (Qiagen GmbH, Hilden, Germany) and stored immediately at -20ºC. The right ovary was kept in 4% formaldehyde. The ampullary regions of both Fallopian tubes were then isolated and opened to release the oocytes. In order to scatter these oocytes they were incubated with hyaluronidase (Hyase, Vitrolife AB, Göteborg, Sweden) for ten minutes over a glass microscope slide. Then, the oocytes were covered with a coverslip and they were counted under a Nomarski interference microscope.

Expression of ELANE, Myeloperoxidase, TIMP3 and RUNX2

Elastase (neutrophil expressed; ELANE) and myeloperoxydase (MPO) gene expressions were used for quantification of neutrophils within the tissue. Tissue inhibitor of metalloproteinase 3 (TIMP3) and runt-related transcription factor 2 (RUNX2) were used as molecular markers of post-ovulation. Total RNA was isolated from the left ovary using Trizol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was made using 2 mg total RNA with 0.5 mg random primer (Promega, Madison, WI, USA) in a total volume of 17 µl. This mixture was denatured at 70°C for 5 min. Then, 0.5 mM deoxy-NTP, 20 U RNAsine, RT-buffer and 200U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) were added to a final

volume of 25 µl. The cDNA synthesis was performed for 60 min at 37°C. Quantitative rtPCR in an ABI Prism 7000 Sequence Detector (Applied Biosystems, Carlsbad, CA, USA) was performed to evaluate the mRNA expression. Commercially available Taqman MGB probes (Applied Biosystems, Carlsbad, CA, USA) were used for the target genes TIMP3 (ID: Rn00441826_m1), RUNX2 (ID: Rn01512298_m1), ELANE (ID: Rn01535456_g1), MPO (ID: Rn01460205_m1) and control (β-actin) gene (ID: Rn01424440_s1). Each amplification reaction consisted of 20 ng cDNA, 1x probe-mix and 1x TaqMan Universal PCR mastermix (Applied Biosystems, Carlsbad, CA, USA) to a final volume of 25 µl. After control of amplification efficiency of the targets genes and control, the relative expressions were presented using the comparative Ct method[17]. Expression of the target gene mRNA were normalized to the expression of the control (β-actin).

-Histology

The right ovary was kept in 4% buffered formaldehyde, dehydrated, embedded in paraffin and cut into 5µm thick sections. Sections were subsequently deparaffinized and rehydrated through graded ethanol, rinsed in distilled water and treated with 0.3% H2O2 and 10% normal horse serum to block endogenous peroxidase and non-specific binding, respectively. Primary antibodies against CD4 (1:100, Abbiotec 250592, Nordic biosite, Täby, Sweden), CD8 (dilution 1:75, Serotec MCA48R, Nordic biosite, Täby, Sweden), CD163 (dilution 1:50, Serotec MCA 342R, Nordic biosite, Sweden) and CD25 (dilution 1:25, Serotec MCA 273R, Nordic biosite, Täby, Sweden) incubated at room temperature for 60 min.

The Envision system (K5007-500 Dako, Copenhagen, Denmark) was used in accordance with the manufacturer's instruction, followed by detection with 3,3´-diaminobenzidine. Thymus and spleen tissue were used as positive controls. For each section, the adjacent section was incubated with 5% BSA/PBS in the absence of the primary antibody (negative control). Sections were examined independently by two observers in a blinded fashion. Five luteinized follicles were randomly chosen per section and stained cells were counted under a light microscope (x400, Leica DM4000B, Leica Microsystems, Madrid, Spain). The mean values for each section were used as individual data points. The intra and inter-observer reliability analysis performed for the different cell subpopulations showed intra-class correlation coefficients ranging from 0.81 (95%CI: 0.68-0.95) to 0.91 (95%CI: 0.81-0.95).

-Statistics

Normal distribution of the data was tested using the Kolmogrov-Smirnoff test. Normally distributed data were presented as means and standard deviations. Not normally distributed data were presented as medians and ranges (between square brackets). Pairwise comparisons between the experimental groups and the control group were done using the Student t-test or the Mann-Whitney U-test when appropriate. P values <0.05 were considered as statistically significant. Calculations were done using SPSS v 20 (IBM Corp, Chicago, IL, USA).

Results

Calcineurin inhibitors levels

The median level of cyclosporin and tacrolimus l were 2128 ng/ml [1578-2892] and 5.5ng/ml [4.6-6.6], respectively. Cyclosporine and tacrolimus levels in the control group were undetectable.

White blood cell counts

White blood cells counts are showed in Table 1. No significant differences were evidenced between groups. The use of calcineurin inhibitors did not modify the different white blood cell subpopulations.

Ovulation rate

Animals in cyclosporine group showed a decreased number (9[0-22], p=0.03) of ovulated oocytes when compared to the control group (22 oocytes [6-39]). No significant difference was found between control and the tacrolimus-treated group (21 [8-41]).

Ovarian neutrophilic markers and ovulation markers

The MPO expression was significantly decreased in the TAC05 and CyA-groups when compared to the control group (p=0.019). No differences between groups were evidenced regarding ELANE expression. The anti-proteolytic activity, measured through RUNX2 and TIMP3 was not significantly different between groups, although RUNX2 expression from TAC group ovaries tended to be downregulated. All rt-PCR results are showed in Figure 2.

Histology

Antibodies against CD163 recognize a specific surface glycoprotein present in macrophages of most kind of tissues, although it does not bind to monocytes. Two main patterns of CD163+ cells were identified in ovaries from this experiment: perivascular (Figure 3A) and within the newly formed corpus luteum (Figure 3B). Such distribution was reproduced in all experimental groups. The total amount of CD163+ cells/corpus luteum did not differ between groups (Control: 14.9±6.7;TAC: 16.2±8.2; CyA: 14.7±5.2; n.s.). CD4 is mainly expressed by T-helper lymphocytes. CD4+ cells were absent in all samples but two (one control and one TAC). In those samples with CD4+ cells, its density was low (0.4 and 0.3 cells/corpus luteum respectively). The CD8 co-receptor is predominantly expressed on the surface of cytotoxic T

cells. CD8-positive cells were present in all groups and no statistical differences were seen between groups (Control: 5.1±3.2 cells/corpus luteum; TAC: 6.2±2.9 cells/corpus luteum; CyA: 7.1±4.0 cells/corpus luteum; n.s.).

Discussion

The greatest development in transplantation surgery was the introduction of effective immunosuppression by the calcineurin inhibitor cyclosporine-A in the 1980s and tacrolimus around 15 years later[1]. Since then there has been a continuous increased utilization of transplantation as treatment of severely compromised organ function, with kidneys being the most prevalent transplanted organ. Moreover, during recent years also vascularized composite tissue transplantation, such as transplantation of the hand and face has reached the stage clinical routine procedures. Today around 18% of transplanted women are of fertile age (according to the Organ Procurement and Transplantation Network -OPTN-, data as of August 31, 2014) and with continuously increasing graft survival and the issue of forming a family is a central issue among the younger age groups of transplanted patients, that usually go back to fully normal lives after transplantation. It is generally perceived that the fertility potential of a female on immunosuppressive medication is compromised although no systematic studies have been carried out on the subject. There exist human case reports and a few research studies in animal models[18,19], that could indicate that the calcineurin inhibitors decreases implantation rate and increases miscarriage rate. To our knowledge there is limited evidence on the effect of immunosuppressive drugs on ovarian function. The biologically central process of ovulation has been compared to a local inflammatory reaction, since a number of inflammatory mediators and also some subsets of leukocytes are central to carry out the tissue remodeling of the extracellular matrix, blood flow changes and changes within the cumulus cells, that are so important for this procedure to continue so that follicular rupture takes place. The intraovarian biochemical changes of ovulation are time- and site-specific and involve various types of molecules with antagonistic effects, essentially metalloproteinases and inhibitors of metalloproteinases, and also different type of cell effectors (theca cells, granulosa and resident leukocytes) that interact promoting matrix remodeling. The final purpose is the extracellular digestion of the stroma surrounding basal lamina and the follicle to this release or mature oocyte containing. In this balancing mechanism, there is a key role metalloproteinases secreted by white blood cells in the ovary residents and inhibitors of metalloproteinases secreted by theca cells, granulosa and fibrobasts (Fedorcsak, et al., 2010).

Like any other inflammatory process, immunosuppressive drugs that modify the function of the different leukocyte subpopulations could potentially regulate ovulation. Secretion of metalloproteinase inhibitors from ovarian tissue cells could also be regulated by immunosuppressants due to the ubiquity of the molecular pathways disrupted by these drugs.

Data in the literature regarding the use of immunosuppressants and ovulation may seem contradictory: while an improvement in fertility parameters is experienced after transplantation of solid organs[3], ovarian dysfunction in solid organ-transplanted patients has also been describe, usually associated to mid-luteal phase defects associated to low progesterone levels[20].

To our knowledge this is the first study up-to-date to systematically evaluate the effect of calcineurin antagonists on ovulation. Our main finding is that cyclosporine-A, but not tacrolimus, decreases ovulation rate when administered for at least two estrous cycles. These differential effects on ovulation by these two types of calcineurin inhibitors were unexpected when we planned the experiment, because the main mechanism of both is the specific inhibition of calcineurin, with the major and common secondary effect being the cessation of interleukin-2 release from T-lymphocytes. However, the differential effects of cyclosporine A and tacrolimus has previously been seen other organs and systems such as the kidney, endothelium or different metabolic systems[21]. Although both cylosporin-A and tacrolimus act on the same molecular pathway, the intermediary proteins mediating their effect differ: while tacrolimus binds to calcineurin through a complex with FKBP-family proteins, cyclosporine binds to calcineurin through a cyclophilin protein complex. The binding sites of FKBP and cyclophilin to calcineurin contain different aminoacids, which induces distinctive conformational changes and subsequently differential activity patterns[22].

It is well-described that leukocytes, especially macrophages and neutrophils, are important in ovulation, since leukocyte supplementation to the perfused ovary preparation would considerably increase ovulation rate and since depletion of neutrophils or macrophages from peripheral blood decreases ovulation rate in vivo[14,23]. The reduction in the ovulation rate evidenced in the CyA-group does not appear to be mediated by any major change in leukocyte infiltration patterns since histological examination of the post-ovulatory ovaries did not find any difference in terms of numbers and distribution of neutrophils, macrophages and lymphocytes in the three experimental groups. Similarly, peripheral leukocyte populations didn't vary between groups. These two facts indicate that CINs do not affect vascular permeability and recruitment of immune cells to the ovary. There exist controversial data in the literature regarding the ability of CNIs to avoid or decrease leukocyte infiltration on different tissues: while most of the available evidence suggest that CINs may affect the chemotaxis of neutrophils exerted by macrophages [10,24,25] and increase endothelial activation resulting in enhanced neutrophil infiltration[26] and macrophage[26] and neutrophil[10] activation, there are studies that fail to evidence any significant effect of CINs on neutrophil, macrophage

or lymphocyte infiltration[27]. These differences between studies could be caused by the different doses and route of administration of CINs, the target organ or the methodology employed.

In this study we decided to use ELANE as the main surrogate marker of neutriphilic infiltration beause it is described in the literature that CINs only alters its expression during hematopoiesis[28]. In mature neutrophils, elastase and myeloperoxidase accumulate in intracellular granules until they undergo exocytosis during neutrophil activation[29]. The presence of neutrophils measured by a surrogate marker for RT-PCR instead of using a histological marker increases the reproducibility of the determination. Indeed, during a parallel experiment using a set of 10 animals neutrophil phenotyping was performed with a battery of commercially available rat antibodies (data not shown). We found a very low specificity for all of them, since other leukocyte subpopulations were stained, mainly macrophages. This would explain some of the discordant results that exist in the literature regarding the role of neutrophils in ovulation[14,30,31].

The expression of MPO, like other enzymes involved in the production of free radical species, can be decrased by the effect of immunosuppressive drugs[32]. MPO expression was reduced both in the group TAC05 as CyA group, indicating that although there is no difference in the number of neutrophils (measured by ELANE), their capacity to produce oxygen-reactive species is diminished.

When we compared the molecular markers of ovulation, although there were no statistically significant differences between groups, a tendency to downregulation of RUNX2 was observed in animals treated with tacrolimus (TAC05). On the other hand, TIMP3 expression was similar between groups. This is consistent with the fact that intracellular signaling pathways involved in TIMP3 regulation seem to be independent from calcineurin[33]. These findings suggest that the detrimental effect on the ovulation rate observed in the group of animals treated with cyclosporine (CyA) is independent of the expression of metalloproteinase inhibitors like TIMP3 by ovarian tissue.

The doses used in this experiment were chosen because they allow obtaining plasma levels of CIN similar to those obtained in a clinical setting of immunosuppression in solid organ transplant. These doses are efficient enough to control the risk of organ rejection in most clinical scenarios. Despite the differences between species, pharmacokinetic CINs profiles in mouse and human models are quite similar[34], as it is their pharmacodynamic profile[35].

The main limitation of this study is that it was designed to assess the main variable effect, the ovulation rate. Given the complexity of the process of ovulation, this experiment allowed studying the mechanisms behind such effect only partially. While one can rule out that the decrease in the ovulation rate caused by calcineurin is due to numerical alterations or infiltration of leukocytes, and also seems unlikely to be justified by imbalances in the production of metalloproteinase inhibitors, there are other factors, such as changes in production of metalloproteinases[28], abnormal vascularization[36] or follicle contractility[37] that should be further explored.

Acknowledgements

The authors thank Ann Wallin for technical assistance and professor Carmen Carda for technical advice. This study was supported by the Swedish Research Council and ALF of Sahlgrenska Academy, Sweden. CDG benefited from a Rio Hortega Grant from the Instituto de Salud Carlos III, Spain (CM09/00063).

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Table 1. White Blood Cells counts and WBC subpopulations in peripheral blood.

Control	Control	Cya	TAC05	p value
WBC (cells/microL)	3063±1560	3178±1246	1958±645	n.s.
Lymphocytes (%)	86±3	77±6	85±6	n.s.
Neutrophils (%)	10±4	13±3	8±6	n.s.
Monocytes (%)	3±1	7±5	5±3	n.s.
Eosinophils (%)	0.1±0.3	0.5±0.5	0.8±0.7	n.s.
Basophils (%)	0.1±0.3	0±0.0	0±0.0	n.s.

Comparisons were done using the Control group as reference. Student's t-test was used. Differences were considered significant if $p<0.05$.

Figure 1. Scheme of the experimental design.

Animals were synchronized for ovulation by injecting 10UI of equine chorionic gonadotrophin (eCG) s.c.; 48h later ovulation was induced by injecting 10UI of human chorionic gonadotrophin (hCG) i.p. and 20 hours after induction the animal was euthanized by cervical dislocation. During the 7 days prior to euthanasia the animals received tacrolimus tacrolimus 0.5mg/kg/day (Tac0.5; n=15); cyclosporin 10mg/kg/day (CyA10; n=15) and normal saline (Control; n=15).

Figure 2. Expression analysis of ELANE, MPO, RUNX2 and TIMP3 mRNA.

Columns represent fold changes using the control group as reference (1-fold change). Bars represent standard deviations of delta-Ct measurements.

Figure 3. Macrophage distribution within the ovaries.

A/ Macrophages disposed around vascular structures (asterisk). B/ Macrophages within luteal cells. (x400)

FIGURE 1

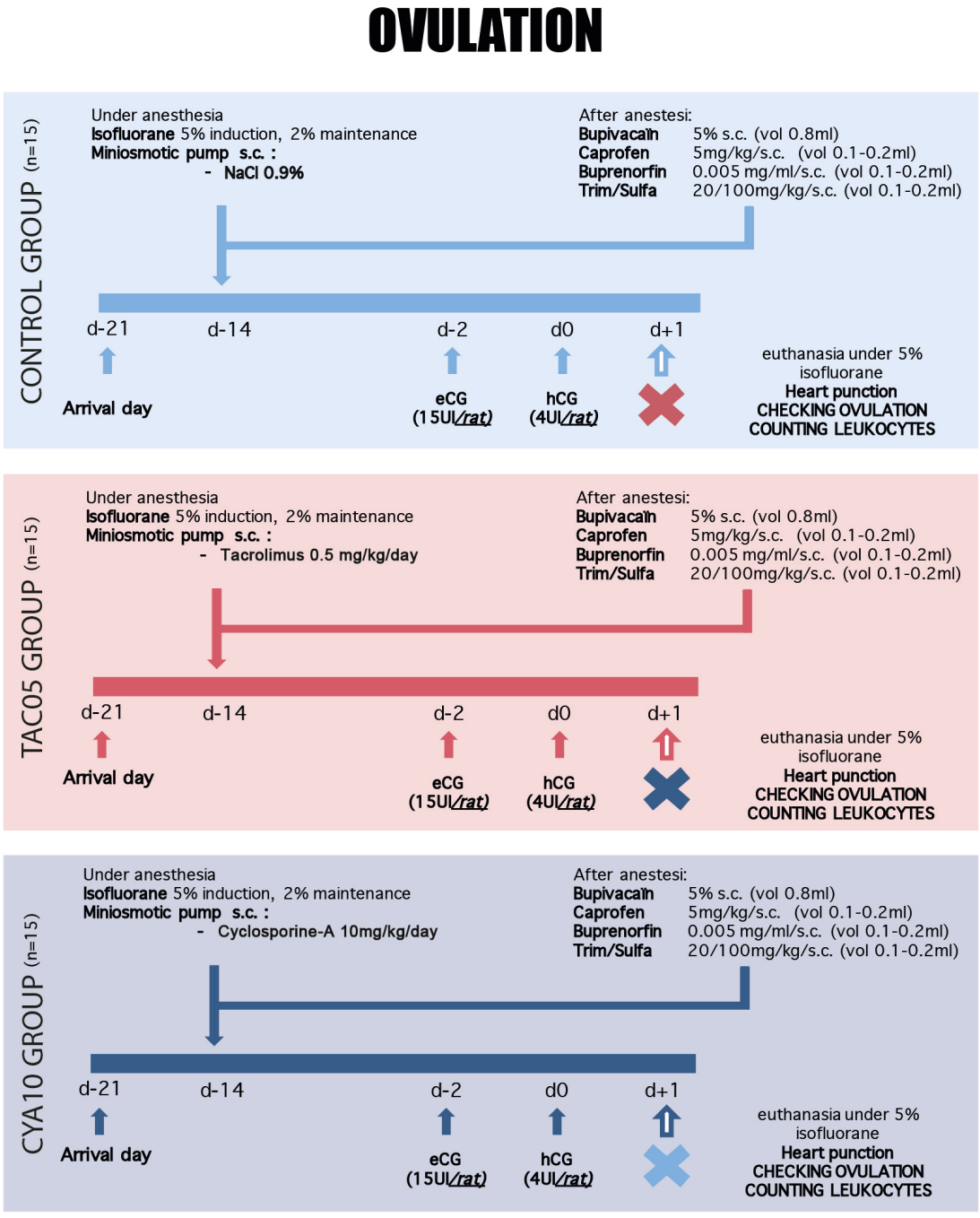

OVULATION
CONTROL GROUP (n=15)
Under anesthesia
Isofluorane 5% induction, 2% maintenance
Miniosmotic pump s.c. :
- NaCl 0.9%
After anestesi:
Bupivacaïn 5% s.c. (vol 0.8ml)
Caprofen 5mg/kg/s.c. (vol 0.1-0.2ml)
Buprenorfin 0.005 mg/ml/s.c. (vol 0.1-0.2ml)
Trim/Sulfa 20/100mg/kg/s.c. (vol 0.1-0.2ml)
d-21
d-14
d-2
d0
d+1
Arrival day
eCG (15UI/rat)
hCG (4UI/rat)
euthanasia under 5% isofluorane
Heart punction
CHECKING OVULATION
COUNTING LEUKOCYTES
TAC05 GROUP (n=15)
Under anesthesia
Isofluorane 5% induction, 2% maintenance
Miniosmotic pump s.c. :
- Tacrolimus 0.5 mg/kg/day
After anestesi:
Bupivacaïn 5% s.c. (vol 0.8ml)
Caprofen 5mg/kg/s.c. (vol 0.1-0.2ml)
Buprenorfin 0.005 mg/ml/s.c. (vol 0.1-0.2ml)
Trim/Sulfa 20/100mg/kg/s.c. (vol 0.1-0.2ml)
d-21
d-14
d-2
d0
d+1
Arrival day
eCG (15UI/rat)
hCG (4UI/rat)
euthanasia under 5% isofluorane
Heart punction
CHECKING OVULATION
COUNTING LEUKOCYTES
CYA10 GROUP (n=15)
Under anesthesia
Isofluorane 5% induction, 2% maintenance
Miniosmotic pump s.c. :
- Cyclosporine-A 10mg/kg/day
After anestesi:
Bupivacaïn 5% s.c. (vol 0.8ml)
Caprofen 5mg/kg/s.c. (vol 0.1-0.2ml)
Buprenorfin 0.005 mg/ml/s.c. (vol 0.1-0.2ml)
Trim/Sulfa 20/100mg/kg/s.c. (vol 0.1-0.2ml)
d-21
d-14
d-2
d0
d+1
Arrival day
eCG (15UI/rat)
hCG (4UI/rat)
euthanasia under 5% isofluorane
Heart punction
CHECKING OVULATION
COUNTING LEUKOCYTES

FIGURE 2

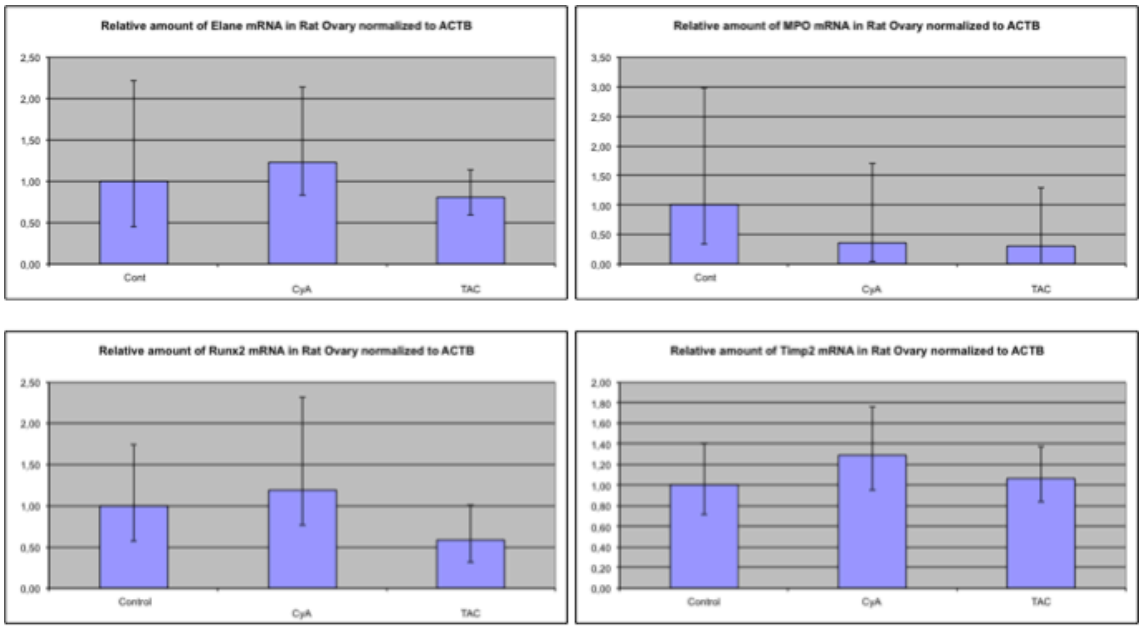
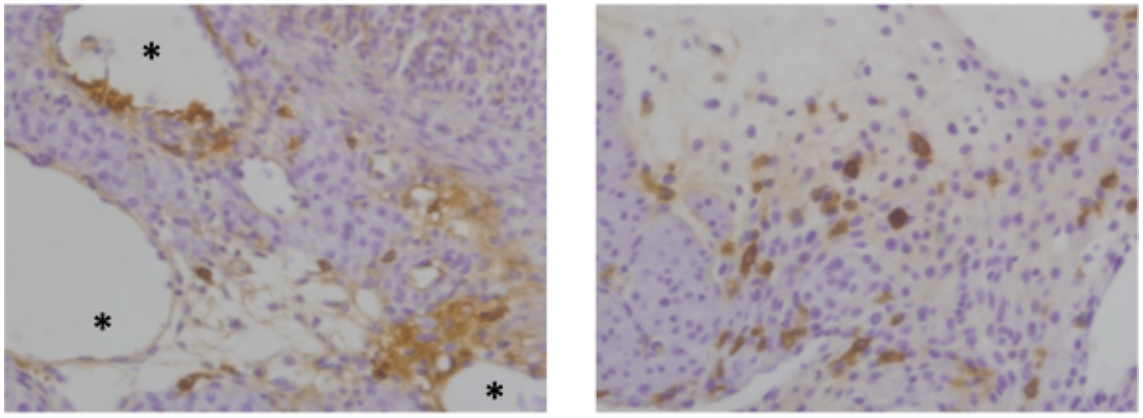

Relative amount of Elane mRNA in Rat Ovary normalized to ACTB
2,50
2,00
1,50
1,00
0,50
0,00
Cont
CyA
TAC
Relative amount of MPO mRNA in Rat Ovary normalized to ACTB
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
Cont
CyA
TAC
Relative amount of Runx2 mRNA in Rat Ovary normalized to ACTB
2,50
2,00
1,50
1,00
0,50
0,00
Control
CyA
TAC
Relative amount of Timp2 mRNA in Rat Ovary normalized to ACTB
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Control
CyA
TAC

FIGURE 3



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human reproduction

ORIGINAL ARTICLE *Gynaecology*

Pregnancy after syngeneic uterus transplantation and spontaneous mating in the rat

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BACKGROUND: Uterus transplantation (UTx) research aims towards the introduction of UTx as a treatment for uterine factor infertility. The rat model is the principal rodent model used and this study aims to assess the potential for pregnancy and to assess effects on pregnancy outcome.

METHODS: Female Lewis rats underwent hysterectomy and received syngeneic uterine transplants (with one horn removed) by end-to-side anastomosis between the common iliac vessels of the recipient and the graft. The graft was placed in an orthotopic position with anastomosis to the upper part of the native uterine horn and vagina to allow for pregnancy by mating. Controls had only one uterine horn removed. Mating and pregnancy frequencies, successful deliveries and pup weight trajectory were compared.

RESULTS: Pregnancy was achieved in rats after UTx with the pregnancy rate, number of pups and growth trajectory of pups being similar to controls. However, numbers of resorbed pregnancies and arrested parturitions were more common in the UTx group.

CONCLUSIONS: A model for orthotopic UTx was developed and pregnancies with live offspring were for the first time demonstrated in the rat model of UTx. The model will be useful in future studies of fertility after UTx.

Key words: uterus / transplantation / syngeneic / fertility / pregnancy

Introduction

Transplantation of solid organs is one of the greatest achievements of modern medicine and the main therapeutic alternative for some patient groups with end-stage organ failure. Substantial advancements in this clinical field have recently allowed for transplantation also of composite tissue (face, hand and forearm) that are not vital for survival but imperative for normal function and quality of life (Dubernard *et al.*, 1999; Devauchelle *et al.*, 2006). Since infertility can interfere substantially with socio-psychological health (Stanton *et al.*, 2002), transplantation of reproductive organs or tissues as infertility treatment may also be encouraged in the future. Untreatable uterine factor infertility exists in women with congenital uterine agenesis and after hysterectomy and may also be present in patients with intrauterine adhesions or large uterine leiomyoma. Despite the advances during the last decades in infertility treatment and transplantation surgery, this group of patients still lack a treatment for their infertility. Transplantation of the uterus has been proposed as a possible temporary curative treatment (Brannstrom *et al.*, 2003, Sieunarine *et al.*, 2005) and an alternative to adoption or IVF surrogacy for these women.

There has been one attempt of uterus transplantation (UTx) in a woman, and although successful in terms of short-term outcome of surgery, a necrotic uterus was removed after 3 months (Fageeh *et al.*, 2002). It is not clear whether the cause of necrosis was related to surgical techniques, rejection or other factors. Nevertheless, UTx may have a place as infertility treatment in the future since large patient groups with uterine factor infertility exist (Sieunarine *et al.*, 2005; Brannstrom *et al.*, 2010). However, before another attempt to transplant a uterus in a woman is performed, the feasibility, safety and benefits of the procedure must be determined by in-depth studies in appropriate animal models.

The aim of any organ transplantation is to re-establish organ function in the recipient, and in UTx, the natural end-point proving organ function is pregnancy with healthy offspring. There are several aspects of UTx that can disturb implantation and pregnancy. The surgery may alter blood flow, lymphatic drainage, structural support and innervation of the uterus so that conditions to establish and

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continue pregnancy become suboptimal. Moreover, drugs used to prevent rejection of the transplant may affect implantation as well as the development of the fetus and future health of the offspring (Groth et al., 2010). We have previously presented a method for heterotopic UTx in the rat (Wranning et al., 2008), showing normal uterine morphology after syngeneic transplantation. The present study presents a modification of this model with orthotopic placement of the uterus graft with the objective to study pregnancy potential after mating.

Materials and Methods

Animals

Female, virgin Lewis rats (weight 170–200 g) were used as uterus donors/recipients and male Lewis rats (Charles River, Sulzfeld, Germany) of proven fertility were used for mating. The animals were housed in controlled conditions (21–23°C; illumination 07:00–19:00 h) with free access to water and standard food. The study was approved by the Animals Ethics Committee in Gothenburg and conducted in accordance with Swedish legislations and the principles and procedures outlined in the 'Guide for the Care and Use of Laboratory Animals' (Commission on Life Sciences, Institute of Laboratory Animal Resources, 1996).

Study design

Rats were randomly allocated for syngeneic uterine transplantation (UTx group) or removal of the left uterine horn (sham group) based on an estimated surgical success rate of 70% in the UTx group (Wranning et al., 2008) and 100% in the sham group. Inclusion criteria for both groups were (i) satisfactory post-operative recovery according to scoring of animal health and (ii) healthy appearance of the uterus at second-look laparotomy 2 weeks after surgery. Included animals were left to recover after surgery for an additional 4–8 weeks and then introduced to males of proven fertility on a one female to one male basis. Mating pairs were kept together until weaning of pups or for 10 weeks. Mating and pregnancy frequency, number of deliveries and pup weights were recorded.

Transplantation surgery

The donor surgery was performed as previously described (Wranning et al., 2008) with modifications for orthotopic placement of the grafted uterus. Briefly, a graft containing the right uterine horn, the common uterine cavity with the cervix and upper vagina plus a vascular pedicle including the arteries and veins from the right uterine vessels up to, and including, the right common iliac vessels, was isolated. To allow for anastomosis of the uterine horn of the graft to the upper part of the recipient uterine horn, the donor uterine horn was divided 7–8 mm from the Fallopian tube after cauterizing the utero-ovarian pedicle at the same level. The uterus was flushed *in situ* through the common iliac artery with 2 ml of cold (4°C) Perfadex preservation solution (Vitrolife AB, Mölndal, Sweden) supplemented with xylocaine (0.4 mg/ml) and heparin (50 IU/ml) and then submerged in cold (4°C) Perfadex during preparation of the recipient.

In the recipient, a simple hysterectomy was performed with dissection and mobilization of the upper third of the vagina from the rectum and the bladder. A titanium clip was placed *en bloc* over the tip of the left uterine horn. On the right side, a 7–8 mm segment of the upper portion of the uterus was preserved for later anastomosis to the uterine graft.

The right common iliac vessels were separated and mobilized up to the aortic/caval bifurcation. Atraumatic vascular clamps (S&T Microsurgery, Neuhausen, Switzerland) were placed on each side of the intended anastomosis sites on the common iliac vessels. The common iliac vessels of the graft were anastomosed to the recipient in an end-to-side fashion using two semi-continuous 10-0 nylon sutures (S&T Microsurgery) on each vessel (Fig. 1a).

The vaginal cuff of the transplant was sutured to the vaginal vault of the recipient by six to seven interrupted 6.0 polyglactin sutures (Ethicon, New Brunswick, NJ, USA). The uterine horn of the graft was anastomosed end-to-end (Fig. 1b) to the remaining cranial uterine segment of the recipient uterus using five to seven interrupted 7-0 nylon sutures (Ethicon).

Control animals were operated under the same conditions as the transplanted rats. Via a midline laparotomy, two titanium clips (Hemoclips; Weck Closure System, Triangle Park, NC, USA) were placed on the left uterine horn; one just caudal to the Fallopian tube and the second one just cranial of the bifurcation from the common uterine cavity. The uterine tissue between these clips was removed. No modifications were made on the remaining horn and only blood vessels and nerves coming through the left infundibulopelvic ligament were excluded. Peri- and post-operative care of all operated animals was performed according to previously described protocols (Wranning et al., 2008).

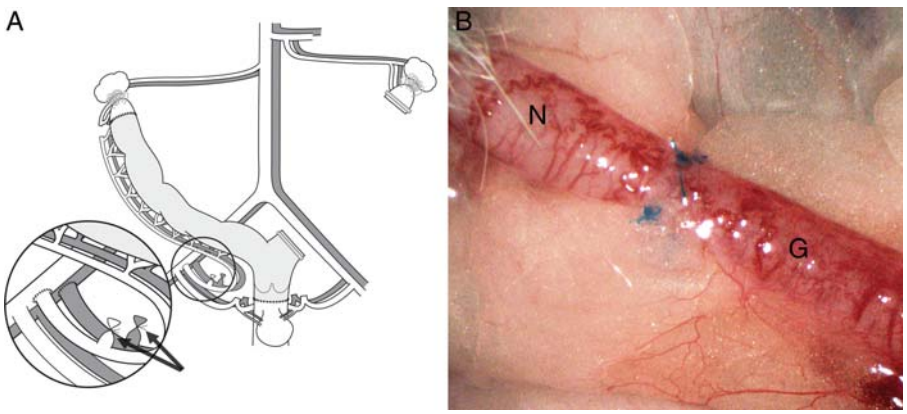

Figure 1 Schematic drawing of the UTx procedure in the rat. (**A**) The grafted uterus is shown in grey and the site of vascular anastomosis of the graft iliac vessels to the recipient common iliac vessels is highlighted in the circle. Arrows mark the ligations of graft vessels caudal of the uterine branch on the iliac vessels. (**B**) Photograph of the anastomosis between the distal end of the native uterine horn (N) and the grafted uterus (G).

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Evaluation of surgical success

All UTx and sham animals were observed during the first 5 post-operative days to assess post-operative recovery. Animals not showing satisfactory recovery according to health scores approximating signs of pain or distress such as piloerection, lethargy and hunched back (Morton and Griffiths, 1985) were excluded.

Two weeks after surgery, all animals were anaesthetized and the laparotomy scar was opened. The uterus transplants (UTx group) or the right uterine horns (sham group) were examined. Animals with uteri displaying signs of thrombosis, constricted uterus–uterus anastomosis or major intestinal–uterine adhesions were excluded.

Assessment of reproductive performance

Four to 8 weeks after uterus evaluation, UTx and sham animals were introduced to males of proven fertility. Females were examined every morning for the first 4 days for indications of mating. Those displaying a vaginal semen plug and/or the presence of sperm in vaginal lavage were concluded as having mated.

Two weeks after detected mating, mated females were weighed and gently palpated to determine pregnancy status. Pregnant females were observed for nesting behaviour and the presence of pups every morning from Days 20 to 24 after mating. The number of pups in a litter was determined on Day 1 after birth to allow time for undisturbed establishment of maternal behaviour. Females displaying palpable pregnancies later during the study period were monitored in the same manner every morning after determination of pregnancy. At the end of the experiments, UTx and sham animals were euthanized and their uteri were examined for signs of resorbed and/or ongoing pregnancies. Pups born by sham and UTx mothers were weighed weekly from Day 7 after birth until 6 weeks of age.

Statistics

The duration of graft cold ischaemia and recipient surgery was compared between included and excluded animals using the Wilcoxon signed-ranks test. Comparisons of mating, pregnancy and conception frequencies between sham and UTx groups were performed using χ^2 test. All tests were two-tailed and a P-value of <0.05 was considered statistically significant.

Results

Outcome of surgery

A total of 27 UTx procedures were performed and 19 animals were included in the study, resulting in a surgical success rate of 70%. The causes for exclusion of eight UTx animals were: death due to bleeding within the first 3 post-operative days ($n = 3$), unsatisfactory post-operative recovery ($n = 1$) and pathological findings [graft thrombosis ($n = 1$), intra-abdominal adhesions ($n = 1$) and constricted uterus–uterus anastomosis ($n = 2$)] during the check-up surgery. The durations (medians) of different stages of the surgery were comparable between included and excluded animals ($P > 0.05$). The median graft cold ischaemia duration was 120 (range: 60–210) and 122 (range: 70–160) min for included and excluded animals, respectively. The median recipient surgery duration was 215 (range: 170–270) and 215 (range: 190–250) min for included and excluded animals, respectively. In the sham group, 19 surgical procedures were performed. The duration of surgery was between 35 and 40 min and surgical success was 100%.

Mating and pregnancy rate

Mating rates during the first oestrus cycle after introduction to the male were comparable in UTx (17 of 19) and sham (16 of 19) animals. Pregnancy rates, determined by palpation on Day 15 after these first matings, were similar between groups: 47% (8 of 17) in the UTx group and 50% (8 of 16) in the sham group. At later time points, an additional three animals in the UTx group and four in the sham group became pregnant (Table I). The overall pregnancy rate for the UTx group (11 of 19; 58%) was not significantly different from that of the sham group (12 of 19; 63%). Mating and pregnancy rates are illustrated in Fig. 2.

Pregnancy outcome

All sham animals that conceived during the first oestrus cycle delivered litters of three to seven pups (median = 3.5 pups) on Days 22–24 of

Table I Breeding performance after UTx and sham surgery.

	Number of animals		
	Utx	Sham	
Animals	19	19	n.s.
Mated (first cycle)	17	16	n.s.
Pregnant animals	11	12	n.s.
Successfully delivered litters	1	8	$P < 0.01$
Total number of pups or fetuses	32	45	
Pups/pregnancy[a]	3 (2–6)	3.5 (3–7)	n.s.
Number of resorptions	6	0	$P < 0.01$

[a]Expressed as median (range).

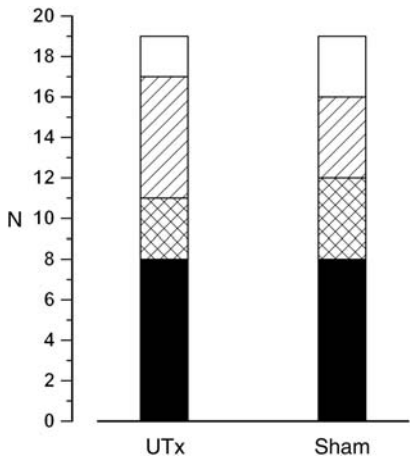

Figure 2 Mating and pregnancy performance in animals after UTx and in sham-operated animals (sham). The black area represents the animals that became pregnant during the first cycle of mating. The squared area represents the animals that became pregnant during subsequent cycles. The striped area represents animals that mated but did not get pregnant. The white area represents the animals that did not mate.

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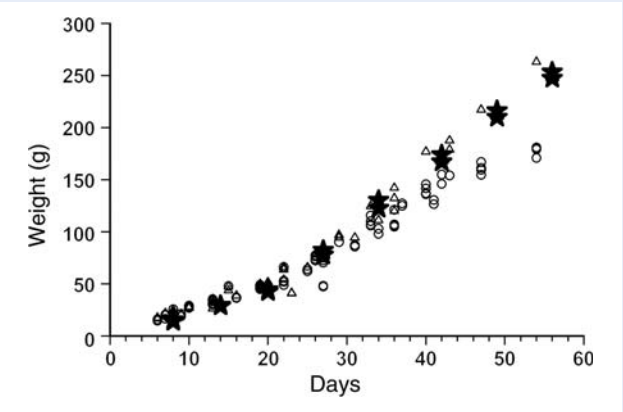

Figure 3 Growth trajectory of offspring of sham and UTx animals. Open triangles represents male pup weights, open circles represents female pup weights and weights of two male pups born from UTx rat are indicated by filled stars.

pregnancy and initiated normal maternal behaviour. Of the eight UTx animals that conceived during the first cycle, seven showed signs of initiation of labour (nesting behaviour, dilation of vagina) on Days 23–25 after mating. One UTx rat delivered two male pups and showed normal maternal behaviour. Two other UTx females gave birth to at least two pups each (concluded by observation through the transparent cage bottom on the morning after delivery) but committed infanticide before the pups were counted. The remaining four pregnant UTx animals displayed signs of distress and labour cessation and were euthanized. At examination, dead pups of full-term size were found in the vagina of the recipient and in the common cavity and right horn of the graft (2–6 pups, median 3).

The UTx and sham females that did not have pregnancies completed from the first cycle of mating were euthanized and examined 10 weeks after introduction to the male. There were four sham and two UTx animals which carried viable fetuses of sizes and weights corresponding to approximately mid-gestational age. Also, two UTx females that previously had been concluded pregnant by palpation and weight gain displayed completely resorbed pregnancies (2 and 4, respectively). Pregnancy outcome is summarized in Table 1 and Fig. 2. The weight trajectory for the only 2 surviving pups (males) born from one UTx rats was similar to the 32 born pups (18 females and 14 males in 8 litters) of sham rats (Fig. 3). Animals that had not conceived during the study period showed uterine gross morphology similar to what had been noted on examination 14 days after surgery.

Discussion

Although UTx research has been ongoing for many years, only a few studies have tested the pregnancy potential of a transplanted uterus. Early experiments studying severance and re-anastomosis of the uterine vessels in the dog reported a limited number of pregnancies (Mattingly *et al.*, 1970; Barzilai *et al.*, 1973). Also, after autologous UTx in the rhesus macaque with neovascularization from the omentum, menstruation was resumed but no pregnancies occurred (Scott *et al.*, 1987). Recently, we reported pregnancies in the sheep after autotransplantation of a uterine-tubal-ovarian graft and spontaneous mating several weeks after the procedure (Wranning *et al.*, 2010). The first pregnancy after transplantation of a uterus from one animal to another was reported in the mouse after syngeneic UTx (Racho El-Akouri *et al.*, 2003a,b). In this mouse UTx model, the graft was placed in a heterotopic position and pregnancies were achieved by transmyometrial embryo transfer (Racho El-Akouri *et al.*, 2003a,b). The present study represents the first report of fertility and live offspring after mating involving any animal that has undergone transplantation of the uterus from one animal to another.

There are several rationales for using an orthotopic rat model instead of the previously reported heterotopic mouse (Wranning *et al.*, 2010) or rat (Wranning *et al.*, 2008) models. The larger size of the rat makes the surgical procedure easier and thus increases model reproducibility (Wranning *et al.*, 2008). The present orthotopic model shows similar animal and graft survival rates as in the previously presented heterotopic mouse (Racho El-Akouri *et al.*, 2003a) and rat (Wranning *et al.*, 2008) models. Also, by this orthotopic model, conception by mating could be achieved. Even though feasible, ovulation induction and embryo transfer in rats are fairly inefficient in inbred rats (Corbin and McCabe, 2002; Popova *et al.*, 2005) and may add confounding factors to the experiment.

An important step in UTx research is to investigate the capacity of an allogeneic uterus transplant to carry a pregnancy. Others have reported that doses of immunosuppressants that are needed to control rejection in rats are, in comparison to that in mice, more in the human range (Kawahara *et al.*, 1980; Giardina *et al.*, 1990). In our mouse model, daily doses of cyclosporine at 20 mg/kg prevented macroscopic signs of rejection during a short study period (Wranning *et al.*, 2007), but increased lymphocytic infiltration indicated that long-term survival may not be feasible even at this high dose. Furthermore, high doses of cyclosporine in mice have been shown to impair implantation rates and fetal survival (Groth *et al.*, 2010).

In the present study, the pregnancy rate was similar between sham and UTx animals, with no difference in the median numbers of pups per pregnancy. However, under the assumption that each uterine horn carries half the litter, the median number of pups in the present model was lower than that reported by other authors in normal animals (Gill *et al.*, 1979; Lord *et al.*, 1999). This may reflect that not only the transplantation procedure but also the insult caused by sham surgery affects the fertility capacity of the uterus. The increased number of resorbed pregnancies in the UTx group indicates that conditions supporting fetal survival after UTx are suboptimal. Whether they are caused by altered blood flow, interrupted lymph drainage, denervation or other changes in the transplanted uterus is unclear and further studies are needed to determine this.

The rate of successful deliveries in the UTx group was markedly lower than in the sham group. In seven UTx rats, parturition was initiated but four of these could not proceed to delivery. Pups found at euthanasia of these females were dead but of appropriate size for gestational age and with no signs of maceration, indicating that the pups died during arrested labour. The initiation and progression of labour in the rat is a complex and incompletely understood process (Zakar and Hertelendy, 2007). Nerve reflexes via the viscerocutaneous branch of the pelvic nerve have been shown to influence parturition (Martinez-Gomez *et al.*, 1998), and in the present study,

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the transplanted uterus was completely denervated. This may provide one explanation for the high percentage of failed deliveries since no such complications were found in the sham group where the uterine and cervical innervation was unmanipulated. Another plausible cause may be scarring at the vaginal anastomosis or at other vaginal sites due to the surgical manipulations, in line with findings that vaginal adhesions may cause dystocia in the sheep (Winter, 2000). In our material, macroscopic examination of the vaginal anastomosis revealed no signs of stenosis, but tests of elasticity and detailed morphological evaluations were not performed. Since the surgical denervation did not seem to affect the duration of gestation, it may be possible to prevent this adverse perinatal outcome by performing a Caesarean section in future experiments using this model.

Two of the rats in the UTx group managed to deliver but committed infanticide within 24 h. Infanticidal tendencies in female rats are uncommon after 22 days of pregnancy (Peters and Kristal, 1983), but female rats in all reproductive conditions are likely to eat stillborn pups or even healthy pups if conditions are stressful (Miley *et al*., 1982). It can be speculated that these females either gave birth to dead pups or that the parturition itself was stressful and induced infanticide. However, since the newborns were not examined immediately after delivery, this remains unclear.

The present study demonstrates that syngeneic, orthotopic UTx allows for conception by mating and pregnancies that are comparable to the control group. This UTx model may be useful in future studies regarding fertility, pregnancy and offspring development after allogeneic UTx under immunosuppression.

Authors' roles

C.A.W.: study design, surgery, animal work, data analysis and writing of manuscript. S.N.A.: surgery, animal work and input in writing of manuscript. C.D.G.: writing of manuscript. M.B.: study design, data analysis and writing of manuscript.

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SHORT REPORT

First report on fertility after allogeneic uterus transplantation

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Abstract

Uterus transplantation may become the first available treatment for uterine factor infertility, which is due to the absence or malfunction of the uterus. Here we describe for the first time pregnancy after allogeneic uterus transplantation, as a proof of concept of uterine function in a transplanted uterus in a standardized animal model (rat) under immunosuppression.

Key words: *Allogeneic, infertility, pregnancy, transplantation, uterus*

Introduction

Transplantation of the uterus may in the future become a treatment for women with uterine factor infertility, which is due to either the absence of the uterus (uterine agenesis/previous hysterectomy) or the presence of a uterus that is not able to carry a pregnancy (intrauterine adhesions/congenital malformation/myoma). Women with uterine factor infertility make up the largest subgroup of infertile women that lack treatment in spite of the great advancements in the clinical area of reproductive medicine. Experimental uterus transplantation (UTx) has developed extensively after the only human trial 10 years ago (1). This human UTx attempt was not successful but stimulated research efforts on several UTx-specific issues such as surgical technique (2–7), uterus rejection (5,8,9) and ischemia–reperfusion events (10–15). Studies have reported pregnancies in non-rejection settings of syngeneic UTx among inbred strains of mice (12,16) and after autologous UTx in the sheep (17). Here we describe the first pregnancy ever reported after allogeneic UTx, with the uterus recipient being under continuous immunosuppression to avoid rejection of the graft.

Material and methods

Female, virgin Dark Agouti (histocompatibility haplotype: RT1av1, RT2b, RT3a, RT7a) rats were used as uterus donors and female, virgin Lewis rats (histocompatibility haplotype: RT1l, RT2a, RT3a, RT7a, RT8b) were used as uterus recipients. Male Sprague–Dawley rats of proven fertility were used for mating. All animals (weight 170–200 g) were from Harlan Nederlands (Horst, Netherlands). All surgical procedures were performed under aseptic conditions and with the aid of an operating microscope (6–40-fold magnification) and bipolar diathermy (Coa Comp, Billdal, Sweden).

The uterus donor animal was anesthetized and given a subcutaneous injection of heparin (1,000 $IU \cdot kg^{-1}$). After disinfection of the abdominal wall a midline laparotomy was performed. The small intestines and appendix were exteriorized from the

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abdominal cavity and protected. To acquire a reasonable operating field around the uterus and its vascular connections, the rectum and bladder were removed from the pelvis after cauterization of their vascular pedicles. The vaginal vessels and descending branches of the uterine vessels were cauterized close to the cervix at the level of the ureters. These were then divided bilaterally after cauterization. The left uterine horn was removed from the remaining uterus after placements of titanium clips at its proximal and distal portions. The cervical/vaginal portion of the intended graft was dissected from the remaining surrounding tissue and subsequently the vagina was carefully divided with the aid of bipolar diathermy at a level 3 mm caudal of the cervix. All the vessels branching from the iliacs between the origin of the uterine vessels and the aorta were carefully ligated (8-0 nylon) and sectioned.

The iliac vessels were separated from each other by gentle dissection from the level of the aortic/caval bifurcation down to the branching of the uterine vessels. The right uterine horn was then cut 2 mm caudally to the utero-tubal junction after placing a titanium clip around the Fallopian tube. Ligatures (8-0 nylon) were placed separately around the left common iliac artery and vein in proximity to the aortic and caval bifurcations and the left common iliac artery was cannulated with a 27G cannula which was secured by an 8-0 nylon ligature. The common iliac vein was then cut just caudally of the ligature at the bifurcation of the vena cava. The uterus was brought out from the abdomen and flushed (0.8 $ml \cdot min^{-1}$) through the iliac artery cannula with cold (4°C) Perfadex preservation solution (Vitrolife AB, Mölndal, Sweden), which was supplemented with xylocaine (0.4 $mg \cdot ml^{-1}$) and heparin (50 $IU \cdot ml^{-1}$). Flushing was performed until clear fluid drained from the venous side of the graft and the graft was then preserved in Perfadex at 4°C until transplantation into the recipient (see below).

The uterus recipient was anesthetized and given a subcutaneous injection of low molecular weight heparin (100 $IU \cdot kg^{-1}$). The small intestines were gently exteriorized, soaked in warm saline/glucose solution and kept at sterile conditions. The bladder was tenderly mobilized from the anterior aspects of the uterine cervix with caution to preserve all the bladder vessels. The uterine arteries and veins were cauterized at their most distal ends, preserving the vaginal branches. After that a hysterectomy was performed. A titanium clip was placed en bloc over the tip of the left uterine horn. On the right side, a 6-mm segment of the upper portion of the uterus was preserved for later anastomosis to the uterus graft. The right common iliac vessels were dissected up to the aortic/caval bifurcation to free them from each other and allow for anastomosis surgery. After vascular clamps had been applied proximal and distal to the anastomosis site, the common iliac vessels of the graft were anastomosed to the recipient in an end-to-side fashion. Vascular anastomosis was accomplished by two hemicontinuous 10-0 nylon sutures for the vein and 8 interrupted 10-0 nylon sutures for the artery. The vagina of the graft was then sutured to the vaginal vault of the recipient using interrupted 6-0 polyglactin sutures. The uterine horn of the graft was anastomosed end-to-end (Figure 1A) to the remaining cranial uterine segment of the recipient uterus, using six interrupted 7-0 nylon sutures which were placed so that they did not enter the uterine cavity. The midline incision was closed in two layers. The median and range of the cold and warm ischemic times were 102.5 [60–150] and 75 minutes [55–105] respectively.

Sham-operated animals were anesthetized as above and operated to remove one uterine horn to create an anatomically similar uterus as compared to the uterine

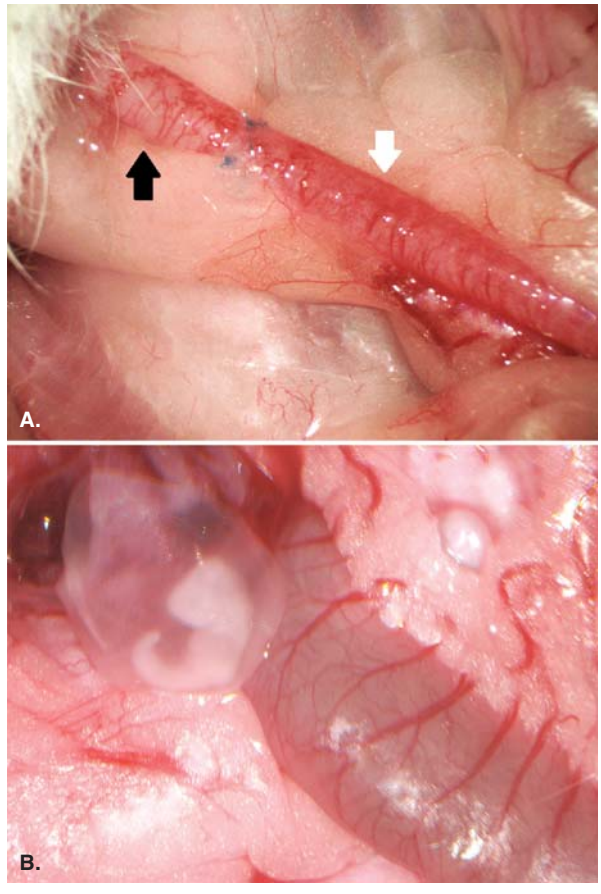

Figure 1. Demonstration of fertility after allogeneic uterus transplantation in the rat. (A) Uterine graft (white arrow) and native tip of uterine horn (black arrow) separated by the anastomosis sutures and with indistinguishable gross morphology. (B) Fetus in its amniotic sac at cesarean section of allogeneic transplanted uterus.

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graft (see above). Thus, the left uterine horn was removed after placements of two titanium clips; one just caudal to the Fallopian tube and the second one just cranial to the uterine bifurcation from the common uterine cavity. The median and range of the total surgery time for the Sham-operated animals were 21 minutes [17–25].

Animals receiving tacrolimus were weighted before surgery. A dose of 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$ tacrolimus diluted in NaCl (final volume 0.2 ml) was injected during surgery. The tacrolimus dose used was chosen according to an initial dose-study (0.2–0.5 $mg \cdot kg^{-1} \cdot day^{-1}$), demonstrating the absence of morphological signs of uterus rejection by this dose (data not shown). The same day a 2ML4 miniosmotic pump (Alzet, Durec Corp, Cupertino, CA) was filled with a solution of tacrolimus prepared individually for each animal. This solution contained between 2.1 and 2.8 mg of tacrolimus depending on the animal weight. The pump was primed in NaCl at 37°C 12 hours prior to its insertion according to the manufacturer's instructions.

Twelve hours after transplantation/sham surgery a skin incision (10 mm) was made in the back, caudally to the neck and the pump was placed subcutaneously in a parasagittal position under isofluorane anesthesia. The final amount of tacrolimus that was released from each pump was estimated to 0.02 $mg \cdot kg^{-1} \cdot h^{-1}$. The pumps were kept for 27 days and then exchanged with a new pump prepared as described above.

For assessment of blood levels of tacrolimus, samples of whole blood (250 µl) were drawn from the aorta at cesarean section. Tacrolimus levels were measured in whole blood by an automated chemiluminescent immunoassay (CMIA) developed for use on the ARCHITECT system (Abbott Scandinavia AB, Solna, Sweden). The coefficient of variation between runs for this assay varies between 1.16 and 1.88%.

The UTx fertility experiments consisted of three groups, with all groups being exposed to fertile males during one estrous cycle between 35 and 38 days after surgical intervention. The first group consisted of eight female rats that had undergone UTx and were immunosuppressed by tacrolimus (UTx-Tac-group). The other groups consisted of sham-operated (excision of one uterine horn) rats treated with either an identical tacrolimus protocol (Sham-Tac-group; $n = 5$) or sham-operated without tacrolimus treatment (Sham-nonTac-group; $n = 6$). All animals underwent cesarean section 17 days after initiation of the mating period. Presence of adhesions, color, size and consistence of the graft were noted according to a previously published scale (3). The anastomosis sites were evaluated regarding pulsation of the artery and refilling of the vein. Pregnancies and fetuses were counted; pregnancy was defined by the presence of at least one identifiable placenta; living fetuses were defined as those showing movements and/or heart beating activity under the surgical microscope and resorbed pregnancies as those having no identifiable fetus or having no living fetuses. Tissue samples were collected from uterus. If the rat was not pregnant transversal biopsies including endometrium, myometrium and serosa were taken randomly. If the rat was pregnant transversal biopsies were taken from the implantation sites and from non-implantation areas. The biopsies were stained with eosin and hematoxylin and analyzed under light microscopy for indications of inflammation according to a previous morphology study of uterine rejection (8).

Although this Dark Agouti-to-Lewis allogeneic rat model has been used in research on other types of organ transplantations, with proven prominent rejection under non-immunosuppressed conditions (18,19), we tested three animals with uterine grafts and no immunosuppression. After 10 days all showed rejected graft with massive necrosis. The surgical model described here is a modification of a rat transplantation model described before (3), even though a series of 10 surgeries were performed in order to avoid learning-curve related problems before starting the experiment.

Results

Before the transplantation experiment started, 10 pre-study transplantation procedures were performed to study the pelvic vascular anatomy of this strain combination in detail and to optimize the procedure. In these 10 trials, perioperative deaths of the donors due to major bleeding occurred during the first three procedures and complete transplantations could not be performed. In pre-study transplantation trials number 4–6, death of the recipients occurred because of major bleeding from the vascular tree of the graft at non-anastomotic sites. The following four pre-study trials were successful in terms of survival of the recipient and graft, when examined 7 days postoperatively.

The tacrolimus levels (median [ranges]) at end of experiment were within the same range in the UTx-Tac-group (11.4 $\mu g \cdot L^{-1}$ [4.6–18]) as in the Sham-Tac-group (9.4 $\mu g \cdot L^{-1}$ [5.7–25]). The pregnancy rates (defined as number of pregnant females/total number of females within group) were 62.5% in the UTx-Tac-group, 60% in the Sham-Tac-group, and 83% in the Sham-nonTac-group. In the UTx-Tac-group all pregnancy sites were present in the uterine graft and none was seen in the small native remnant of the uterine horn. The medians [ranges] of

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fetuses (Figure 1B) per pregnant rat were 1 [0–3] in the UTx-Tac-group, 1 [1–5] in the Sham-Tac-group and 3 [1–4] in the Sham-nonTac-group. Resorbed pregnancies were seen in four of five pregnant rats of the UTx-Tac-group (median 1 resorbed pregnancy [0–3]) and in one (1 resorbed) of four and one (1 resorbed) of five of the Sham-nonTac-group and Sham-Tac-group, respectively. None of the uteri showed macroscopic signs of thrombosis and/or rejection. Uterine morphology (gross/light microscopy) was similar in uteri of all groups with no signs of inflammation or necrosis in any group.

Discussion

Although there are more than 15,000 human live births reported after allogeneic transplantation of organs such as the kidney, liver and heart (20), it is important to ascertain that the delicate physiological processes of implantation and pregnancy can occur as well in a transplanted uterus with novel vascular connections and immunosuppression to prevent rejection of that specific organ. The demonstration of pregnancy in this allogeneic UTx model is a central proof of concept of UTx and thereby an essential step in the research toward clinical application of UTx in the human. The occurrence of resorbed pregnancies and a low pregnancy rate in the allogeneic uterine grafts suggest that both UTx and tacrolimus treatment negatively affects the implantation/pregnancy rate. Advances in immunosuppression treatment or novel modalities to induce tolerance of a transplanted organ may minimize these problems in the future.

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ORIGINAL ARTICLE: REPRODUCTIVE BIOLOGY

Pregnancy after allogeneic uterus transplantation in the rat: perinatal outcome and growth trajectory

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Objective: To investigate whether allogeneic uterine grafts in a rat model, with tacrolimus immunosuppression, can harbor pregnancies that result in offspring with normal postnatal growth.
Design: Experimental animal study.
Setting: Obstetrics and gynecology department at a university hospital.
Animal(s): Lewis rats as uterus donors and Piebald-Virol-Glaxo rats as recipients.
Intervention(s): Animals were allocated to one of the following three groups: allogeneic uterus transplantation with end-to-side anastomosis to the external iliac vessels and immunosuppression with tacrolimus (UT+Tac; n = 10); sham surgery and immunosuppression with tacrolimus (Sham+Tac; n = 10); or sham surgery (Sham; n = 10). The rats were subsequently introduced to male rats with proven fertility and in the event of resulting pregnancy cesarean sections were performed on day 22 of pregnancy.
Main Outcome Measure(s): Graft viability, fertility rate, perinatal death, birth weight, postnatal birth trajectory.
Result(s): Pregnancy rate was higher in the control groups (70% Sham and 80% Sham+Tac) than in the transplanted group (50% UT+Tac), although these differences did not reach the significance threshold. There were no differences between groups regarding number of living pups or neonatal deaths. Pups born from UT+Tac mothers had birth weights similar to external control animals from our breeding colony (BC): UT+Tac males 6.2 ± 0.2 g, UT+Tac females 5.5 ± 0.6 g, BC males 5.8 ± 0.2 g, BC females 5.2 ± 0.3 g; n.s. Evaluation of uteri and placentas of pregnant animals revealed a somewhat reduced vascular density in both tissues in the UT+Tac group, and that was not seen in the Sham+Tac group.
Conclusion(s): Allogeneic uterus transplantation and immunosuppression with tacrolimus is compatible with normal pregnancy and perinatal outcome in a rat model. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Rat, uterus, transplantation, allogeneic, immunosuppression, pregnancy, offspring

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Despite the significant progress accomplished in reproductive medicine during the past decades, women with absolute uterine-factor infertility (AUFI) still lack any infertility treatment. This group of patients includes those lacking a uterus (congenital/hysterectomy) or having a nonfunctional uterus (Asherman syndrome, major uterine malformation) (1).

Uterus transplantation (UT) has been proposed as a method to treat women with AUFI and to achieve genetic motherhood in settings where surrogacy is not a valid option. However, before clinical introduction of UT, several aspects should be evaluated thoroughly in nonhuman animal models to optimize the procedure before clinical introduction and to ascertain that the procedure is safe. Uterus transplantation is a novel surgical procedure, and the introduction should follow the recently launched IDEAL concept (2), where nonhuman research is proposed to be a fundamental base before any surgical procedure enters the human clinical arena.

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The importance of nonhuman research and training to gain success in an experimental human setting was illustrated by the extensive research primarily conducted in rats before performance of the first full face transplantation in the human (3).

Human UT is, like face transplantation, a quality of life–enhancing type of transplantation, rather than a life-saving procedure. Therefore, in such a situation it is of uttermost importance to perform thorough preclinical preparations, because there is not only the recipient who is affected by the risks involved but also the prospective child, that will develop in the grafted uterus.

Our group initiated research on UT in experimental animal models more than 10 years ago, and we have extensively studied several aspects of UT, such as surgical techniques, tolerance to ischemia-reperfusion events, rejection patterns, and immunosuppressive protocols (4–8). However, the important and natural end point of UT is the delivery of healthy offspring, and this aspect has been studied only after syngeneic UT in mice and rats (9, 10). The important added factor in an allogeneic setting is the immunogenicity of the nonself uterine graft, with the need of effective immunosuppression to avoid rejection. Our group demonstrated already in 2010 the first pregnancies after allogeneic UT experiments performed in the rat model (11). However, the main outcome of that study was pregnancy and not achievement of viable offspring, so evaluations were done at pregnancy day 17 and not at term.

Large databases exist on pregnancies in humans with solid organ transplants under immunosuppression. Higher rates of low birth weight, preterm birth, hypertension, and preeclampsia were noted (12). However, it is not clear whether these obstetrical complications were secondary to immunosuppression or to the underlying disease, because a complete population-based study with reliable data also from pregnancies before transplantation showed a similar increase of these complications in the pregnancies occurring before transplantation (13).

It is clear that it is of great concern to evaluate the outcome of allogeneic UT, where adverse effects on the pregnancy and fetus may be mediated not only by the immunosuppressive medication but also by factors such as altered uterine blood supply and denervation. In the present paper, we present the first data on the perinatal outcome of offspring from allogeneic uterine grafts, evaluating the effect of the anatomic modifications and the immunosuppression caused by the transplantation procedure on their growth phenotype.

MATERIALS AND METHODS

Animals

Virgin Lewis (LW) rats (histocompatibility haplotype RT1l, RT2a, RT3a, RT7a, RT8b) were uterus donors, and virgin Piebald-Virol-Glaxo (PVG) rats (histocompatibility haplotype RT1c, RT2b, RT3a, RT6a, RT7a, RT8b) were used as recipients. All female rats weighed 170–200 g. Male Sprague-Dawley (SD) rats of proven fertility were used for mating. All animals were provided by Harlan Nederlands and were fed ad libitum with standard pelleted food under 12-hour dark-light cycles. The study design and animal procedures were approved by the Animals Ethics Committee in Gothenburg.

Study Design

PVG female rats were randomly allocated to three different experimental interventions: 1) allogeneic UT under immunosuppressive treatment with tacrolimus (UT+Tac group; n = 10); 2) surgical removal of the left uterine horn under immunosuppressive treatment (Sham+Tac group; n = 10); and 3) surgical removal of the left uterine horn without any immunosuppressive treatment (Sham group; n = 10).

Only animals showing a satisfactory postoperative recovery and healthy appearance of the uterus at second-look laparotomy 2 weeks after surgery were included in the experiment. Nine weeks after surgery, the female rats were introduced to male rats of proven fertility and the mating and pregnancy rates, numbers and sex of pups, weights, and perinatal mortality rate were recorded and compared.

Two breeding colonies were used as external control groups for weight: 1) pups born from PVG females (BC; n = 52); and 2) pups born from PVG females undergoing an identical immunosuppressive treatment as the experimental groups during pregnancy (BC+Tac; n = 86). Comparisons were made between pups born from the three experimental groups as well as between pups born from the UT+Tac group compared with those of the breeding colonies (Supplemental Fig. 1, available online at www.fertstert.org).

Surgical Procedures

The transplantation, sham surgeries, and postoperative care of the animals have been described in detail elsewhere (14). Briefly, a graft containing the right uterine horn, the uterine cervix, the uterine pedicle, and a vaginal rim was retrieved from the Lewis donors. The unilateral vasculature pedicle encompassed the arteries and veins of the uterine vessels, the internal iliac vessels, and the major part of the common iliacs. The harvested uterus was kept under cold ischemia while the PGV recipient was prepared by both hysterectomy and clearance of the anastomosis sites on the right common iliacs. Vascular anastomosis was performed in an end-to-side fashion to the external iliac vessels of the recipient by continuous 10-0 Prolene sutures. The vaginal rim of the graft was sutured to the vaginal vault of the recipient, and the abdomen was closed by standard two-layered procedure.

Immunosuppression

The immunosuppression protocol was based on monotherapy with tacrolimus, according to our observations in nonpregnant rats with the allogeneic uterine grafts (15). The dose was higher during the initial 8 weeks and then reduced to 50% of the starting dose. This was kept during the remainder of the experimental period, which included pregnancy. This immunosuppression reduction after the initial post-transplantation period is common in clinical solid organ transplantation.

In the immunosuppressed groups, an initial dose of 0.4 mg/kg tacrolimus (Prograf; Astellas Pharma) was

delivered by subcutaneous injection just after transplantation/sham surgery. A 2ML4 miniosmotic pump (Alzet; Durec Corp.) was immediately placed subcutaneously in the back of the rat. The pump was loaded with a tacrolimus solution in 0.9% NaCl, adjusted for the weight of the animal, to release 0.4 mg/kg/d of tacrolimus. This pump was subsequently replaced after 4 weeks by a similar pump and then every 4th week by a 2004-model miniosmotic pump (Alzet) adjusted to deliver 0.2 mg/kg/d of tacrolimus until the end of the experiment. In the nonimmunosuppressed group, the pumps were filled with the 0.9% NaCl vehicle only.

Mating, Pregnancy, and Delivery

Nine weeks after surgery (1 week after the insertion of the third miniosmotic pump) the female rats were introduced to the male rats of proven fertility in a 2 female:1 male fashion. They were kept together for a complete estrus cycle, and mating was assessed every morning by vaginal plug examination. If pregnancy occurred, the day in which the plug was visualized was considered to be pregnancy day 0. All of the females underwent abdominal palpation 2 weeks after withdrawal from the male. If pregnancy was suspected, a cesarean section was performed on pregnancy day 22. When pregnancy was not obvious, a midline minilaparotomy was performed to rule out the possibility of a single-order pregnancy or an early miscarriage in which the uterus would not be easily detectable by palpation. Animals that did not get pregnant during the first mating cycle underwent a second cycle of mating during the subsequent estrus cycle.

Macroscopic Evaluation of Uteri and Pups after Cesarean Section/Laparotomy

All uteri were macroscopically evaluated during cesarean section or laparotomy (if not pregnant) with the use of a validated scale accounting for color, texture, and size of the uterus, patency of the uterine vessels, vascularization of the myometrium, and presence of abdominal adhesions (16).

The numbers and sexes of live pups, as well as the numbers of dead fetuses and implantation sites were noted. The placentas were placed in formaldehyde for histologic analysis. The live pups were introduced to foster mothers.

Histologic Evaluation of the Graft/Sham-Operated Uteri

Uterine and placental samples were systematically taken at the time of cesarean section/minilaparotomy and fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Tissue sections were made every 5 μm. One-half of the sections were stained with eosin and hematoxylin and the other half were subjected to immunohistochemistry. The slides were deparaffinized in xylene and rehydrated before antigen retrieval. Sections were blocked with 5% normal goat serum in 0.01 mol/L Tris-buffered saline solution (pH 7.6) with Triton X-100 (TBST) in a humidity chamber for 1 hour at room temperature (RT) before incubation with polyclonal rabbit antiendothelin A receptor (1:100, ab85163; Abcam) or polyclonal rabbit antiendothelin B receptor (1:100, ab39960; Abcam) overnight at 4°C. Sections were washed with TBST (3×, 5 min) at RT before incubation with goat anti–rabbit IgG coupled to Alexa 488 (1:250; Invitrogen) for 1 hour at RT in a humidity chamber. Secondary antibody was removed and tissues washed again with TBST (3×, 5 min) before mounted with fluorescent Vectashield (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI). Slides were viewed on an Axiovert 200 confocal microscope (Carl Zeiss) equipped with a laser-scanning confocal imaging LSM 510 Meta system (Carl Zeiss) and photomicrographed. Background settings were adjusted from examination of negative control specimens. Controls for nonspecific staining have been described elsewhere (17).

Tacrolimus Levels

Peripheral blood samples, to measure tacrolimus levels, were taken by incision of the distal edge of the tail twice: on day 17 of pregnancy and at the time of cesarean section/minilaparotomy. Tacrolimus levels were measured in whole blood by an automated chemiluminescent immunoassay (CMIA) developed for use on the Architect system (Abbott Scandinavia). The intra-assay coefficient of variation was $<1.9\%$.

Weight of the Pups

Pups were weighed at birth and then every week until the age of 16 weeks, with the use of a precision scale (Classic series; Mettler-Toledo). Low birth weight (LBW), adjusted for age and sex, was defined as the <10th percentile of the weight of the pup population born from normal animals (PVG females × SD males) that were mated in the same room, under the same housing conditions, and at the same time as our experiment was carried out (i.e., pups from BC group).

Statistics

Variable values are presented as median and ranges for continuous variables (except for the weights of the pups, which are expressed as means and standard deviation) or absolute values and percentages for categoric variables. Normality of the data was tested with the use of normality plots and Shapiro-Wilk and Kolmogorov-Smirnov tests. Data following a normal distribution were compared with the use of the analysis of variance test. Nonnormal distributed data were compared with the use of the Kruskal-Wallis test. Categoric variables were compared with the use of the chi-square test and the Fisher exact test when needed. Differences showing $P<.05$ were considered to be significant.

RESULTS

Surgical Outcomes

To achieve group sizes of 10 animals per intervention group, 10 rats were operated in the Sham group, 10 in the Sham+Tac group, and 14 in the UT+Tac group. There were different causes for exclusions in the latter group: one animal died during the first postoperative day, the cause being intra-abdominal hemorrhage secondary to leakage from the anastomosis site; another animal died from anesthetic

complications while a miniosmotic pump was being replaced 4 weeks after transplantation; one rat presented a necrotic uterus during second-look laparotomy 2 weeks after UT; and the fourth animal was excluded because of extensive left-side hydronephrosis that was found at second-look laparotomy. All animals primarily included in the Sham group and the Sham+Tac group had normal appearances at second-look laparotomy.

Fertility Outcomes

Pregnancy rates were higher in the control groups with sham surgery (70% in Sham group, 80% Sham+Tac group) than in the UT+Tac group (50%), but these differences did not reach the significance threshold. There were no differences between groups regarding number of living pups or neonatal deaths (Table 1). Litter size was significantly larger, although showing a great range, in animals of the BC+Tac group (13 [range 0–14]) than in the other experimental groups.

Macroscopic Appearance of Uteri

Twenty-nine out of the 30 rats of the three experimental groups showed macroscopically normal uteri at ocular examination at the time of cesarean section. One rat in the Sham+Tac group showed a whitish pale uterus with increased size (microscopic examination is described below in the "Histologic Evaluation" section). This was one of the nonpregnant animals with two cycles of mating attempts. Mild intra-abdominal adhesions were seen between the intestines and uterus in 4 out of 10 UT+Tac rats. Macroscopic signs of thrombosis or rejection were not present in any animal of the UT+Tac group.

Tacrolimus Levels

Tacrolimus levels were similar between the tacrolimus-treated animals during mid-pregnancy (UT+Tac 1.88 ± 0.60 ng/mL, Sham+Tac 1.77 ± 0.49 ng/mL, BC+Tac 1.92 ± 0.45 ng/mL; n.s.) and at the time of cesarean section/laparotomy (UT+Tac 1.50 ± 0.20 ng/mL, Sham+Tac 1.63 ± 0.63 ng/mL, BC+Tac 1.71 ± 0.48 ng/mL; n.s.).

TABLE 1

Fertility and perinatal outcomes after allogeneic uterine transplantation.

Outcome	UT	Sham+Tac	Sham	BC	BC+Tac
Mating (1st cycle)	9/10	10/10	8/10	10/10	10/10
Mating (2nd cycle)	4/6	5/5	6/6	–	–
Pregnancy rate	5/10	8/10	7/10	8/10	8/10
Live pups					
Mothers	5/5	8/8	7/7	7/8	6/8
Pups	5 (1–7)	2.5 (1–7)	1 (1–5)[a]	5.5 (0–14)	13 (0–14)[a]
Stillborns					
Mothers	1/5	3/8	0/7	2/8	2/8
Pups	0 (0–1)	0 (0–1)	0	0 (0–7)	0 (0–4)
Neonatal deaths					
Mothers	1/5	2/8	1/7	1/8	1/8
Pups	0 (0–2)	0 (0–2)	0 (0–2)	0 (0–1)	0 (0–1)

Note: Values are presented as raw data for dichotomous variables and median (range) for continuous variables. For each perinatal outcome, "mothers" represent the number of rats whose offspring presented the event/number of pregnant rats; "pups" represent the median (range) number of pups per litter size presenting the event. UT = uterine transplantation; Tac = tacrolimus; BC = breeding colony.

[a] Sham vs. BC+Tac: $P<.05$.

Díaz-García. Offspring after allogeneic uterus transplantation. Fertil Steril 2014.

Histologic Evaluation

Microscopic examination of the uteri of the nonpregnant rats showed similar endometrial and myometrial patterns in all experimental groups, except for one animal in the Sham+Tac group that showed evident fibrosis in all uterine compartments. Thrombi were present in both arterial and venous vessels in that animal. Variations of different phases of the estrus cycle were seen in uteri from nonpregnant animals, suggesting that these animals were normally cycling. Evaluation of uteri and placentas of pregnant animals revealed no major disruptions of placental structures in any of the three experimental groups. The vascular density in both the subendometrial location and the spongiotrophoblast had a clear appearance of being lower in the UT+Tac group compared with the Sham and Sham+Tac groups (Fig. 1). No differences were found in pattern or expression of endothelin receptors A and B when uteri and placentas were compared (Fig. 2).

Weight and Weight Progression of the Offspring

Male pups from UT+Tac mothers had higher birth weight than male offspring from the Sham group (6.2 ± 0.2 g vs. 5.3 ± 0.1 g; $P<.001$) but not compared with those from the Sham+Tac group (5.9 ± 0.6 g). There were no differences between experimental groups regarding birth weight of female offspring (UT+Tac 5.5 ± 0.6 g, Sham+Tac 5.3 ± 0.3 g, Sham 5.1 ± 0.5 g).

Compared with control animals of the breeding colony, both males and females born from UT+Tac mothers had similar birth weights (UT-Tac males 6.2 ± 0.2 g, UT-Tac females 5.5 ± 0.6 g, BC males 5.8 ± 0.2 g, BC females 5.2 ± 0.3 g; n.s.). The proportion of low-birth-weight pups did not differ significantly between experimental groups (UT+Tac 10.5%, Sham 7.7%, Sham+Tac 11.3%) or with the BC group (10% by definition).

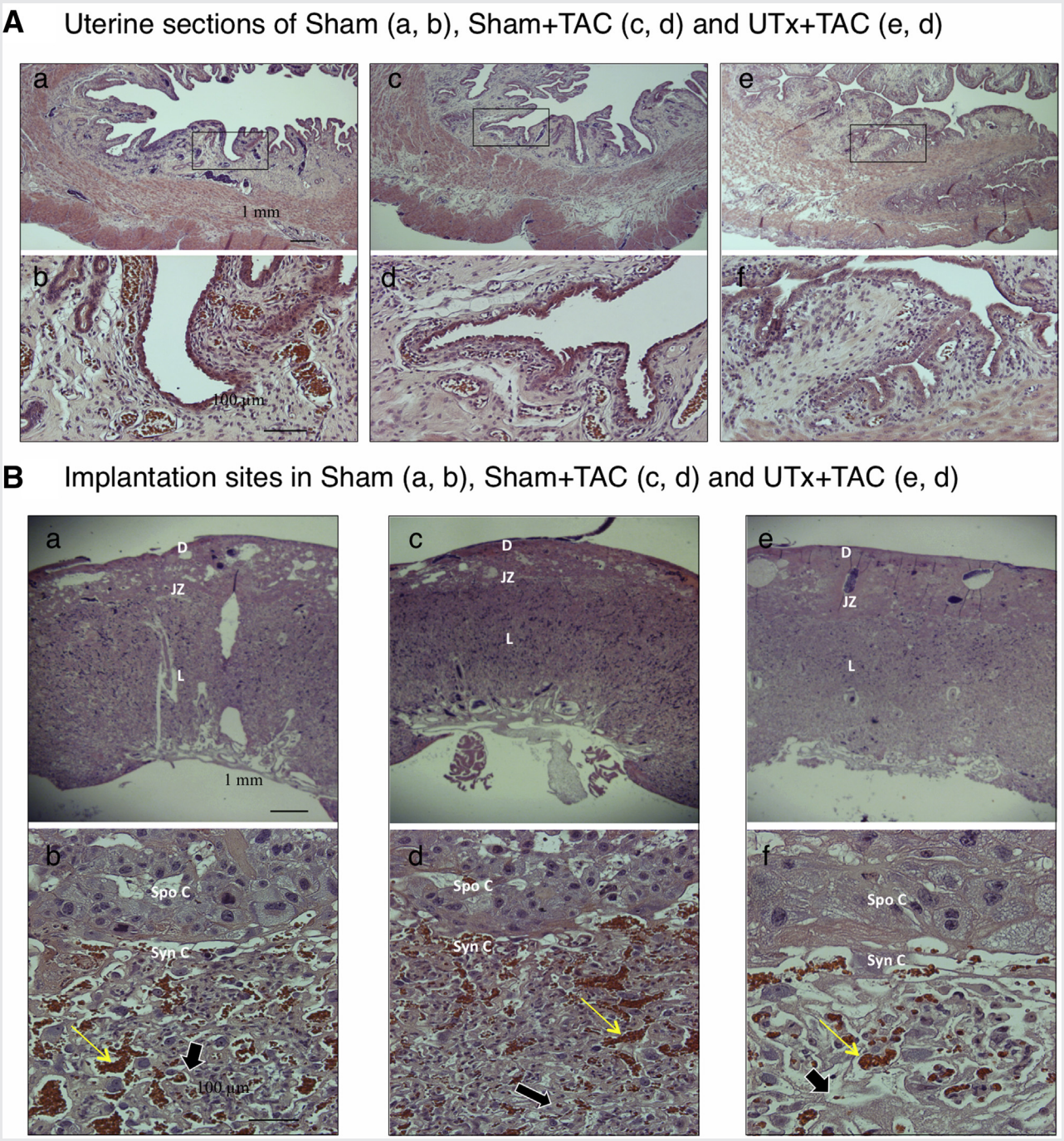
Growth trajectories until postnatal week 16 are shown in Figure 3. No weight differences were seen at any time point between female offspring from the different groups. The male offspring from the UT+Tac groups showed a trend to be heavier compared with the other experimental groups and to the external breeding colony control groups. This difference was significant from postnatal week 10 (Fig. 3B) but not at earlier time points.

DISCUSSION

This study evaluates for the first time postnatal outcome in an allogeneic UT setting. The main finding is that the birth weight of offspring is not affected. However, vascular density of the uterus was affected, although placental weights were unchanged. Moreover, the postnatal growth of male pups from the UT+Tac group was slightly accelerated after postnatal week 10.

Fertility and perinatal outcomes after UT have already been reported by our group as well as by other groups in

FIGURE 1

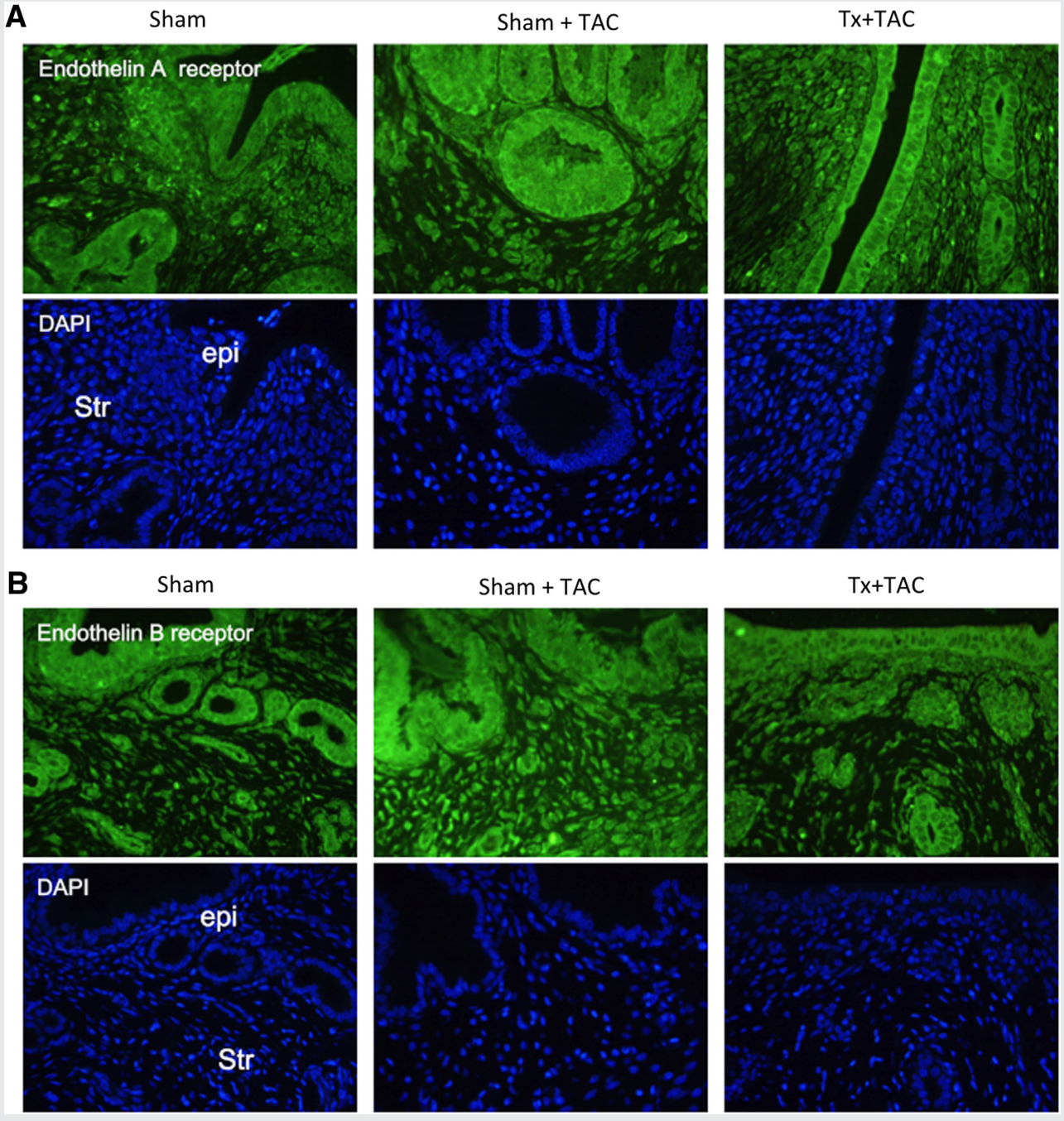


Histologic sections of uteri from pregnant rats. Hematoxylin and eosin staining. A lower vascular density is seen in the uterine transplantation (UTx) group. Such decreased vascularization pattern is noticed in both (**A**) nonimplantation sites and (**B**) implantation sites. TAC = tacrolimus; L = labyrinth zone; JZ = junctional zone; D = decidual basalis cells; Syn C = syncytiotrophoblast cells; Spo C = spongiotrophoblast cells. *Yellow arrows*: maternal vascular compartment; *black arrows*: Fetal capillary space.

Díaz-García. Offspring after allogeneic uterus transplantation. Fertil Steril 2014.

nonrejecting settings in rodents (9, 14, 18) and large animals (10, 19). In the syngeneic setting of UT, where immunosuppression is not needed, experiments, evaluating pregnancy as the main outcome, are mostly testing the effects of surgery, altered vascular connections, altered position of the uterus, and denervation of the uterus. The studies carried out in the syngeneic mouse model with a heterotopically positioned uterus demonstrated normal birth weight and normal growth trajectory over 8 weeks after full-term pregnancies that had been initiated by embryo

FIGURE 2

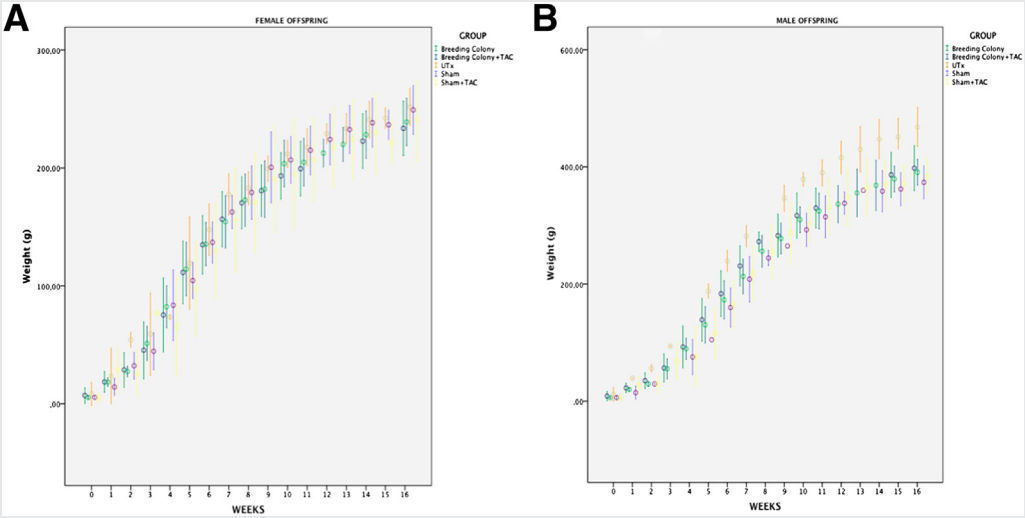


Uteri with immunofluorescence staining. (**A**) Endothelin A and (**B**) B receptors show a similar distribution pattern between groups, being expressed in both epithelial (epi) and stromal (Str) compartments. DAPI = 4′,6-diamidino-2-phenylindole; TAC = tacrolimus; Tx = uterine transplantation.

Díaz-García. Offspring after allogeneic uterus transplantation. Fertil Steril 2014.

transfer (9). Such results were later replicated in the rat orthotopic and syngeneic UT model, with pregnancies after spontaneous mating (14). In an autologous transplantation setting in sheep, pregnancy was seen in three out of five transplant recipientss (three out of four animals that mated), but the offspring were evaluated only at birth (10). There is also a case report of live offspring after autologous UT in a primate species (cynomolgus macaque); a cesarean section was urgently performed after diagnosis of placental abruption in pregnancy day 143, but resuscitation of the neonate was not performed and consequently no follow-up was performed on that offspring (19). It should be pointed out that to date, this is the only reported pregnancy with live offspring in any primate species.

FIGURE 3



Comparison of weight evolution between pups from the different experimental groups and the two breeding colonies. The *y*-axis presents the weight in grams, and the *x*-axis presents the age in days. Data are presented for (**A**) female and (**B**) male offspring. Abbreviations as in Figure 1.

Diaz-García. Offspring after allogeneic uterus transplantation. Fertil Steril 2014.

In an allogeneic setting, the effect of immunosuppression to avoid organ rejection is an additional nonphysiologic factor that has to be taken into account. There exist two reports in the literature describing pregnancies after allogeneic UT. In 2010 we published experiments in which Lewis rats received uteri from Dark Agouti donors (11). In that particular experimental design, involving monotherapy with tacrolimus, pups were delivered at day 17 of pregnancy (term pregnancy is 20 days) because the major end point was pregnancy rate. Subsequently, a study of allogeneic UT in sheep, with the use of immunosuppression by cyclosporine, described a single preterm lamb born at day 138 of pregnancy (20). Pregnancies in a nonhuman primate species after allogeneic UT have not yet been demonstrated.

The fertility rate in the UT+Tac group of the present study is similar to that of our previous study in an allogeneic rat UT setting (11). Animals in all three experimental groups of the present study had smaller litter sizes than animals of the breeding colony. A plausible explanation of this is the 50% reduction of potentially implantable endometrium, because all animals of the experimental groups had undergone surgical excision of one uterine horn. The animals of the breeding colony groups (BC and BC+Tac) were intact and had normal bicornuate uteri.

Overall, our data regarding pregnancy rates suggest that the implantation potential of a transplanted uterus is not impaired compared with a native one. Another fact supporting this hypothesis is that offspring born from UT+Tac mothers did not show lower birth weights or a higher rate of low birth weight than offspring from the Sham group or the breeding colonies, suggesting that the blood flow entering the graft is enough to support a normal pregnancy. If the pregnancy-related increase in uterine blood flow to the graft, through the single uterine-iliac arterial vascular tree, in combination with the side-positioned outflow from the native common iliacs, would be restricted or decreased to a large extent, the birth weight should have been reduced. Experimental data in several animal species indicate a direct relationship between uterine blood flow and size of offspring. In a model with experimentally lowered uterine blood flow during pregnancy in the rat, the reduced uteroplacental perfusion affected birth weight of the offspring, regardless of whether the reduction was initiated before (day 12) or after (day 14) the initiation of the physiologic substantial fetal growth increase (21).

The morphologic analysis in the present study showed that microvessel densities were decreased in both the uterus and the placenta of the UT+Tac group. Importantly, this was not seen in the Sham+Tac group, which would indicate that it is an effect solely of the surgical tissue trauma, denervation, and altered vascular supply/drainage after UT, or of those factors in combination with added negative influences of tacrolimus. Despite the change in microvascular density, fetal growth was not affected. This could be explained by the fact that the complex and not completely understood (22) mechanisms controlling uteroplacental blood flow are multifaceted and that compensatory mechanisms exist and neutralize any possible negative effect on fetal growth by the impaired microvascularization.

In the present study we also examined the expression of endothelin and its receptor in the uterine tissue. Endothelin is a potent vasoconstrictive substance released by endothelial cells. Several studies suggest a link between altered expression of endothelin and its receptor in the uteroplacental unit and obstetrical complications. The local levels of endothelins are increased up to three times in uteroplacental disorders such as preeclampsia and fetal growth restriction (23, 24). Endothelin receptors in patients with preeclampsia and low-birth-weight offspring are usually down-regulated in uteroplacental circulation. This probably reflects a mechanism

aiming to maintain the maternal blood supply to the fetus (22). In the present study, such down-regulation was not seen in any placental sample from either group, indicating that endothelin-mediated responses in the uteroplacental circulation are not dysregulated by UT or immunosuppression.

In summary, this is the first report evaluating postnatal outcome of offspring after allogeneic UT in any species. The data showing normal growth up to an age well after sexual maturation, despite pregnancy in a uterine graft under immunosuppression, indicates that allogeneic UT may be regarded to be a safe procedure in terms of perinatal outcomes. However, one has to bear in mind that these results come from a rodent species and that it could not directly be extrapolated to larger animals, including nonhuman primates and humans.

Moreover, further research is needed to ascertain that the offspring after allogeneic UT also develop normally at older ages and that the phenotype is normal when examining multiple aspects. Such investigations are underway in our research group; the cohort of pups from this study were followed for 24 months, which represents two-thirds of the life of a rat, and aspects such as behavior, metabolism, urinary function, and fertility have been recorded for phenotype characterization. The subsequent analysis of these data will provide further information to characterize the safety of UT.

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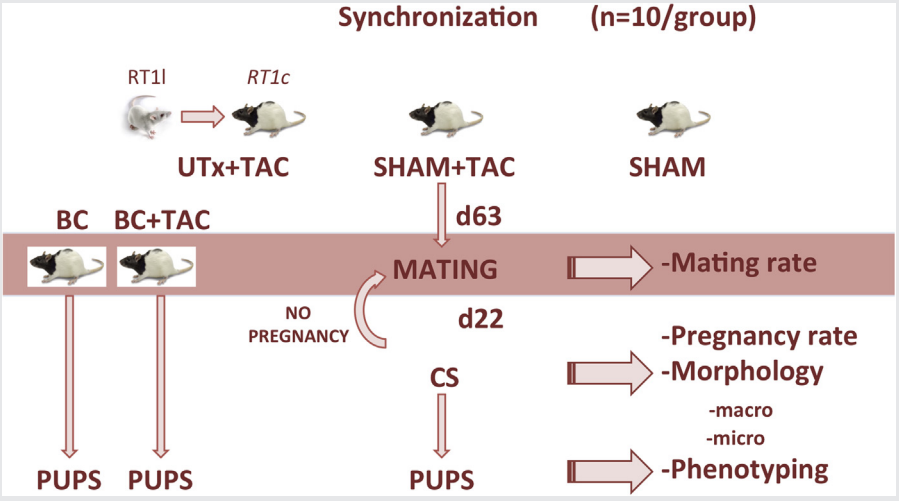
SUPPLEMENTAL FIGURE 1



Schematic illustration of the study design. UTx+TAC = allogeneic uterine transplantation and immunosuppression by tacrolimus; Sham+TAC = surgical removal of the left uterine horn and immunosuppression by tacrolimus; Sham = surgical removal of the left uterine horn without any immunosuppressiion; BC = external breeding colony; BC+TAC = external breeding colony and immunosuppression by tacrolimus. All animals under immunosuppression were administered tacrolimus at a dose of 0.4 mg/kg/d for 8 weeks and then 0.2 mg/kg/d until the end of the experiment.

Díaz-García. Offspring after allogeneic uterus transplantation. Fertil Steril 2014.

Pregnancy outcome after maternal solid organ transplantation: nationwide population-based cohort study over four decades

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Pregnancy outcome after maternal solid organ transplantation: nationwide population-based cohort study over four decades

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Running title: Pregnancy outcome after maternal organ transplantation

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Key words: transplantation; pregnancy, preeclampsia; preterm birth; immunosuppression

Abbreviations: MBRN, Medical Birth Registry of Norway, NRR, Norwegian Renal Registry, SGA, small-for-gestational-age, LBW, low birth weight, OR, odds ratio, AOR, adjusted odds ratio, CI, confidence interval

"What this paper adds"

What is already known on this subject:

Data issued from case reports, case series and voluntary registries suggest that pregnant women transplanted with solid organ present increased rates of adverse perinatal outcomes such as pre-eclampsia, preterm delivery and small for gestational age. Given the source of such information, incomplete data collection and selection bias is likely to occur.

What this study adds to the subject:

Our findings suggest a somewhat lower risk of preeclampsia and preterm delivery than previously reported in the literature. This study also suggests that the observed increase in adverse perinatal outcome could be explained by the underlying disease motivating the transplantation and not by the transplantation itself. From a methodological point of view the strength of the present study is that being a nationwide population-based study, the cohorts include pregnancies from virtually all transplanted women and non-transplanted women taking place during the study period, so that the risk of introducing bias is minimized.

Summary statistics:

A total of 195 deliveries after transplantation were identified, in which the majority occurred during 1990-2009. The absolute risks of preeclampsia (21.6%, 42/195) and preterm delivery (34.0%, 66/195) were lower than previously reported. In comparison to the general female

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population, transplanted women had increased risk of preeclampsia (AOR: 6.5, 95%CI: 5.0-9.1), preterm delivery (AOR: 7.1, 95%CI: 5.3-9.6) and cesarean section (AOR: 9.7, 95%CI: 7.2-13.0). The infants had increased risk of small-for-gestational-age (AOR: 2.5, 95%CI: 1.7-3.5), low birth weight (AOR: 2.7, 95%CI: 1.7-4.1), stillbirth (AOR: 2.9, 95%CI: 1.2-7.0) and perinatal death (AOR: 3.2, 95%CI: 1.5-6.7), but the risk of congenital malformations was not increased (AOR: 1.6, 95%CI: 0.8-3.3). No increase in adverse perinatal outcome was found when comparing pregnancies before transplantation with pregnancies after transplantation.

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ABSTRACT

Objective: To compare the risk of adverse perinatal outcome in pregnant women before and after transplantation and to that of non-transplanted women.

Design and setting: Cross-linked population based nation-wide retrospective cohort study including 130 pregnant women transplanted with a solid organ and 2511202 non-transplanted pregnant women. The study period was 1967-2009. All pregnancies were registered in the Medical Birth Registry of Norway (MBRN). Transplanted patients were identified through the Transplantation registry from Oslo University Hospital. Perinatal outcomes are systematically reported to the MBRN. Adjusted risk estimates for adverse perinatal outcomes were obtained using logistic regression analysis.

Results: A total of 195 deliveries after transplantation were identified, in which the majority occurred during 1990-2009. The absolute risks of preeclampsia (21.6%, 42/195) and preterm delivery (34.0%, 66/195) were lower than previously reported, but an increasing preterm (<37 and <34 weeks) delivery proportion over time was found. In comparison to the general female population, transplanted women had increased risk of preeclampsia (AOR: 6.5, 95%CI: 5.0-9.1), preterm delivery (AOR: 7.1, 95%CI: 5.3-9.6) and cesarean section (AOR: 9.7, 95%CI: 7.2-13.0). The infants had increased risk of small-for-gestational-age (AOR: 2.5, 95%CI: 1.7-3.5), low birth weight (AOR: 2.7, 95%CI: 1.7-4.1), stillbirth (AOR: 2.9, 95%CI: 1.2-7.0) and perinatal death (AOR: 3.2, 95%CI: 1.5-6.7), but the risk of congenital malformations was not increased (AOR: 1.6, 95%CI: 0.8-3.3). No increase in adverse perinatal outcome was found when comparing pregnancies before transplantation with pregnancies after transplantation.

Conclusions: Transplanted women have a high risk of adverse pregnancy outcome when compared to the general population. Notably, our findings suggest a somewhat lower risk of preeclampsia and preterm delivery than has previously been reported from registries on pregnancies after transplantation. Such increase in adverse perinatal outcome could be

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explained by the underlying disease motivating the transplantation and not by the transplantation itself.

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Introduction

In the early era of solid organ transplantation, great concerns about possible teratogenic effects of immunosuppressive agents were raised [1] and the general advice was to avoid pregnancy.[2-4] Today, more than 50 years after the first birth from a transplanted women,[5] planned pregnancies in transplanted females are common, with more than 14000 births being reported up to 2006 in the three transplantation-pregnancy registries that cover USA and Europe.[3] Increased rates of adverse perinatal outcomes such as preeclampsia, preterm delivery and small for gestational age were reported in the data from these registries. Importantly, no such study has found any increased risk of fetal malformation after in utero exposure to standard immunosuppressive drugs.[3] The current knowledge of possible adverse effects of immunosuppressants, in relation to pregnancy and perinatal outcomes, and the resulting guidelines for antenatal management of these patients are mainly based on data accumulated from single case reports, case series or studies from voluntary registries,[3] in which incomplete data collection and selection bias is likely to occur.

The objectives of the current study were to assess the risks of congenital malformations and adverse outcomes of pregnancy, delivery and the perinatal period in pregnancies after maternal solid organ transplantation in Norway, with data obtained from two large and complete databases spanning a period of 40 years.

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Material and methods

Data source and study population

A national cross-linked population-based cohort study was designed, using data from the Medical Birth Registry of Norway (MBRN) and the Transplantation registry from Oslo University Hospital, from 1967 to the end of 2009. The MBRN was established in 1967 by the Norwegian Directorate of Health and was the first complete national medical birth registry in the world. It is based on compulsory notification of all live births and stillbirths, initially (1967-2001) registered from gestational week 16 and since 2002 from week 12. A standardized notification form is used to collect data on demographic variables, maternal health before and during pregnancy, previous reproductive history, complications during pregnancy and delivery as well as pregnancy outcomes. The form is completed by the midwife or the attending obstetrician and was unchanged from 1967 up to and including 1998, with the exception of the addition of Apgar scores in 1978. In 1999, a new and more detailed form was introduced, in which smoking habits and ultrasound-based estimated dates of parturition were included. All records in the MBRN are matched with the files of the Central Person Register to ensure medical notification of every newborn in Norway[6] and to collect dates of deaths.

All transplanted males and females were identified from the Transplantation registry of Oslo University Hospital. Oslo University Hospital has since 1969 kept complete records of all transplanted patients in the country. Renal transplantation was the only type of transplantation conducted in Norway during the initial 15 years of the study period (1969-1984). Our analysis included all female patients that received a transplant during the period from January 1969 up to and including 2009. Transplanted males were identified to exclude any pregnancy emerging from couples with both parents being immunosuppressed, and thereby to exclude

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possible influences carried over from the male. Linkage between the Transplantation registry from Oslo University Hospital and the MBRN was performed by utilizing the unique personal identification number given to every Norwegian citizen. Information about type of transplanted organ and date of transplant were collected from the Transplantation registry, whereas data on pregnancy, delivery, perinatal complications and congenital malformations were obtained from the MBRN.

Definitions

Preeclampsia, induction of labor, operative vaginal delivery (forceps or vacuum delivery) and cesarean section has been registered in the MBRN since its start. The following sources of the MBRN were available to estimate gestational age: 1) last menstrual period (registered from 1967) and 2) expected date of parturition according to ultrasound (registered from 1999). The latter source was used if available and preterm delivery was defined as a delivery occurring <37 completed weeks of gestation. Small for gestational age (SGA) was defined as an infant birth weight by gestational age that was below the 10th percentile according to a national birth weight standard based on data from the MBRN.[7] This criterion is in accordance with common clinical practice.[7] Similarly, and in accordance with the same national birth weight standard, large for gestational age was defined as infant birth weight by gestational age above the 90th percentile. Low birth weight (LBW) was defined as infant birth weight below 2500g. Perinatal mortality included fetal death in utero (stillbirth, from week 23+0) and death within seven days after birth. Congenital malformations are registered as ICD-8, 9 and 10 codes in the MBRN and were defined as major malformations, i.e., not including insignificant diagnoses in accordance with a previously used definition.[8] Data were divided into four time periods (1969-1979, 1980-1989, 1990-1999 and 2000-2009).

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Statistical analysis

Proportions were compared using the chi-square test; continuous variables were compared using the Student-t test and p-values below 0.05 were considered statistically significant. Risk estimates were calculated as odds ratios (OR) and adjusted odds ratios (AOR) with 95% confidence intervals (CI) using logistic regression analysis (IBM SPSS Statistics 20.0.0 SPSS Inc, Chicago, IL, USA). Adjustments for maternal and paternal age, parity, and year of delivery were performed if not stated otherwise. Couples in which both mother and father had been transplanted were excluded. Pregnancies from mothers who had undergone organ transplantation after giving birth were excluded from comparative general population. Perinatal outcomes of patients giving birth both before and after transplantation were compared using the most recent birth before transplantation and the first one after transplantation. In these patients, McNemar's test was used to compare proportions of events, conditional regression analysis was performed using a propensity score controlled for the same confounding factors controlled when comparing to the general population (R software, Matching -v.4.8-3.1- and Ime4 -v. 1.0-4- packages).[9] Associations were considered statistically significant if the CI did not include 1.

Ethical review board approval

The regional ethics committee in Southern Norway approved the study (REK Sør-Øst A 2010/2028a-2).

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Results

A total of 2511202 births were reported in the general population during the time period (1967-2009) and 195 births were from 130 mothers after transplantation (1969 to 2009). The majority of pregnancies in the transplanted group (Fig. 1) occurred among renal transplant patients (170 pregnancies, 87%). The other types of organ transplantations were kidney+pancreas (7 pregnancies, 3.6%), kidney+liver (1 pregnancy, 0.5%), liver alone (13 pregnancies, 6.7%) and thoracic organs (4 pregnancies, 2.1%). Number of deliveries by time period for the 195 deliveries is illustrated in Fig. 1. The vast majority (80.5%) of the 195 deliveries occurred during the last two decades (after 1990).

Demographic characteristics of all pregnancies are shown in Table 1. The ages of both the mother and the father of the maternal transplant group were higher, but the parity was lower than in the general population.

Numbers, percentages and crude and adjusted odds ratios for pregnancy and perinatal outcomes are shown in Table 2. Almost 22% of all pregnancies after organ transplantation developed preeclampsia, which is a more than 6 times increased risk (AOR: 6.5, 95%CI: 5.0-9.1) as compared to the general population.

The proportion of preterm delivery before 37 weeks among transplant patients was 34%, as compared to 6.1% in the general population (AOR: 7.1, 95%CI: 5.3-9.6).

At separate analysis of the renal transplant patients, we found almost similar overall proportions for preeclampsia (20.6%) and preterm delivery (34.1%) as in the total (n = 195) transplant population. The proportions of preterm delivery (<37 and <34 gestational weeks) after renal transplantation increased significantly over time (p-trend= 0.017 and 0.002,

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respectively: Figure 2). The proportions of preeclampsia in this renal transplant subgroup for the four time periods were: 14.3, 19.4, 11.7 and 29.2%, respectively (p-trend=0.10).

The overall proportion of cesarean section was 62.4 % and the corresponding adjusted odds ratio was 9.7 (7.2-13.0). This proportion was 71.4% during the time period 1967-79 and 64.4% in 2000-09.

In a sub-analysis of the type of cesarean section (emergency or planned) with recorded data from during the time periods 1990-1999 and 2000-2009, the proportion of planned cesarean sections increased both among the transplanted group and in the general population (transplanted: 45.7% vs 60.9%, p<0.001; non-transplanted: 37.5% vs 45.4%, p<0.001).

Numbers, percentages and crude and adjusted odds ratios for adverse perinatal outcomes and major malformations are shown in Table 3. Infants of transplanted women were at increased risk of SGA (AOR: 2.5, 95%CI: 1.7-3.5) and of LBW (AOR: 2.7, 95%CI: 1.7-4.1). They were also at increased risk of stillbirth (AOR: 2.9, 95%CI: 1.2-7.0) and perinatal death (AOR: 3.2, 95%CI: 1.5-6.7). The risk of major malformations was not significantly increased (AOR: 1.6, 95%CI: 0.8-3.3), but numbers were small. Of the 8 registered major malformations, 6 occurred among 87 deliveries during 2000-2009, giving an overall proportion of 6.9%. This malformation rate was 8.3% when assessing patients with renal transplants alone.

We also analyzed the births after transplantation in comparison to the births of the same women before transplantation. In transplanted patients, 1192 transplanted mothers gave birth to 2260 newborns before transplantation. After controlling for confounding factors, no increase in adverse perinatal outcome was found when comparing pregnancies before transplantation with pregnancies after transplantation (the exact numbers and risk estimates

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are added in the supplementary appendix in Tables 1 and 2). To avoid mixing independent and dependent observations, perinatal outcomes were further compared in patients giving birth after and before transplantation (Table 4). This part of analysis only included the most recent birth before transplantation and the first birth after transplantation in patients having more than one birth prior to or after transplantation. In this analysis births after transplantation showed a lower incidence of all adverse perinatal outcomes, although no statistically significant differences were evident, except for cesarean section, which was more prevalent after transplantation (OR: 3.2, 95%CI: 1.0-10.1).

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Discussion

The present study represents a unique complete nationwide population-based study to assess the outcomes of pregnancy and neonates of immunosuppressed mothers with solid organ transplants. The major findings of the study are that of major (> 6-fold) increments of preeclampsia and preterm delivery rates among females with organ transplants.

The number of births worldwide from transplanted mothers are today likely to be more than 20000, based on the fact that more than 14000 births were reported in 2006 to the three major voluntary registers of pregnancy in transplanted patients and that these registries mainly cover Europe and the USA.[3] There would most likely exist underreporting into these registries. Furthermore, the recent introduction of new types of organ and composite tissue transplantations, the development of assisted reproductive technologies and the increased survival of transplant patients will most likely lead to an increased birth rate among immunosuppressed women that have undergone transplantation.

In our study, encompassing a period from the late 1960s up until today, it was found that more than 80% of all pregnancies after solid organ transplantation in Norway occurred after 1990 and 87% of these pregnancies were after renal transplants. This was expected, as rather few patients were transplanted during the early time periods of the study and renal transplantation alone represents the majority of all organ transplants in most countries.

Frequent pregnancy complications, as seen in the present study, confirm earlier findings of a generally increased risk of several pregnancy-associated problems that may affect both the transplanted mother and her infant, when compared to the general population of pregnant

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women. Our finding of 22% preeclampsia among the transplanted females is somewhat lower than previously reported in large studies of data from voluntary registries [3]. The preeclampsia proportion for all transplanted patients found in data from the UK Transplant Pregnancy Registry was 36% and in the US National Transplant Pregnancy Registry it was 29%[10]. A validation study of preeclampsia in the MBRN showed that registered preeclampsia corresponds well with medical records.[11] A recent study on trends for preeclampsia in Norway also concluded an increase in the condition over the period 1967-98, due to an improved case ascertainment over time.[12] A predisposing factor for preeclampsia in renal transplant patients is that a large proportion of these patients experience both hypertension and proteinuria after transplantation, which may make them more prone to develop preeclampsia. There is little reason to believe that the findings of the present study, with comparatively lower preeclampsia rates, were due to underreporting, at least not in the latter periods. Also, the compulsory nature of the MBRN should lead to valid data. The overall preeclampsia proportion in this study is similar to data from a Swedish population-based study of births after maternal organ transplantation.[13] Interestingly, our data shows that the proportion of preeclampsia does not increase over time and it could thus not explain the observed increase seen in preterm delivery.

A striking difference in our results compared to previous reports from voluntary registry data is the much lower preterm delivery proportion that we report.[3 10 14-18] The overall rate of preterm delivery in our material of transplanted mothers was 34% and, in a sub-analysis of time trends, revealed an increase over time. Previous studies with data mostly accumulated from voluntary transplant pregnancy registries from Europe, UK and the US have indicated rates of preterm delivery of around 50%, but with some variations depending on type of organ transplantation. The very high odds ratio of preterm delivery (7.1) in our material, despite the

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relatively lower rate of preterm birth, is due to a very low preterm delivery proportion in the general population (6.1 %), which is about half of what is reported in the general US population.[19] This difference in overall preterm delivery proportion may also be partly dependent on differences in routines for delivery of high-risk pregnancies.

Differences may exist in preterm rate depending on the mother's specific type of transplanted organ. Earlier studies have indicated an increased rate of preterm delivery among renal transplant patients as compared to liver or heart transplant patients.[3] However, the proportion of preterm delivery in our data was similar when examining renal transplant patients alone as compared to the entire group of transplanted women.

When analyzing births before and after transplantation, we could not detect any significant increase in adverse perinatal outcomes after adjustment for confounding factors. These data are in line with those published by Kallen and coworkers,[13] suggesting that the morbidity status of the patients could be the underlying cause of adverse outcomes. Moreover, when comparing births within the same patient before and after transplantation, we found a lower rate of preeclampsia, preterm birth, growth restriction problems and perinatal mortality, indicating that the transplant may have a protective effect on both fetus and mother. This could be explained by a better general health of the women after transplantation, which of course may be partly counterbalanced by possible negative effects of immunosuppression. It bears mentioning that, in our series, the incidence of adverse pregnancy events was lower than in the previous publication, which compared pregnancies before and after transplantation.[13]

The risk of being subjected to cesarean section in the transplanted patients was high. Interestingly, the rate of planned cesarean section increased over time in transplanted women. This increase was also seen in the general population. A plausible explanation for the

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increased planned cesarean section rate among the transplanted patients is that this subgroup would follow the general trend, although it is not necessarily medically motivated.

Strengths and limitations

The completeness and the compulsory data collection of the MBRN and the Transplantation registry at Oslo University Hospital enabled identification of all transplanted and non-transplanted women and men in the country becoming parents during the last four decades. This is a complete nationwide population-based study on pregnancy, delivery and perinatal outcome in transplanted females. Norway has a national health care system, where priority assignment of patients is determined by the parliament in terms of laws and regulations.[20] This should guarantee that the patients were treated uniformly during their pregnancies, regardless of their geographic location within Norway. The Norwegian Society for Obstetrics and Gynecology has, for almost 20 years, had national guidelines for management of all pregnancies in the country, contributing to equitable care of pregnancies and its complications throughout the country. Norway was ranked second highest among OECD countries in 2010 (after the United States) in terms of health care spending per capita (www.oecd.org). All in-patient care is provided free of charge[21] and public resources accounted for 85.5% of health care spending in the country (www.oecd.org).

Despite strengths of the study, we are aware that there are some limitations. The majority of transplanted patients (87%) were renal patients and consequently firm conclusions considering pregnancies of mothers with other types of organ transplants are impossible. The various congenital malformations are rare events, which also makes it difficult to draw conclusions about risks related to any specific malformations, as previous studies have encountered. However, there was no increased risk for congenital malformations overall, which is reassuring news for all transplanted women planning for pregnancy. It should also be

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pointed out that the fetal immunosuppression exposure during the entire pregnancy may induce more subtle changes in the child, which is not detected at birth and may well be related to small changes in the immune system of the child. Thus, there exist case reports suggesting increased prevalence of autoimmune disease later in life among those born from transplanted mothers.[22]

The low number of patients and events when comparing births before and after transplantation in the same patient did not allow us to control for confounding factors when the regression analysis with propensity score was applied. As each patient was her own control and births were successive and close in time, we do believe that potential confounders would play little or no role in modifying risk estimates.

Conclusions

The completeness of the data presented in the current study provides near accurate risk estimated for pregnancy complications in transplanted females. The preterm and preeclampsia rates were increased 6- and 7-fold over the general population. The SGA rate was increased 2.5 fold. More important, no increased rate of major malformations was found. It is concluded that pregnancies of transplanted mothers should always be regarded as high risk.

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Competing interest statement

All authors have completed the ICMJE uniform disclosure form at http://www.icmje.org/coi_disclosure.pdf and declare: C.D. was a Rio Hortega Grantee from Carlos III Health Institute (Grant number CM09/00063); no financial relationships with any

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organisations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work.

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Contributorship statement

M.B., N.H.M. and C.D.G. were responsible for the design of the study; C.D.G. performed the statistical analysis; A.V.R, A.F., T.L. and O.G. contributed to variable selection and data extraction from the transplantation registry. The manuscript was redacted by C.D.G., N.H.M and M.B. with specific input from all other authors. All authors read and approved the final version of the manuscript submitted to BMJ.

Transparency statement

This manuscript is an honest, accurate, and transparent account of the study being reported; no important aspects of the study have been omitted; and that no discrepancies from the study as planned have occurred.

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Table 1. Demographic characteristics of the general population and pregnancies after solid organ transplantation, Norway 1969-2009.

	General population	**After transplantation**	
Fathers			
Age group			
<19	31782 (1.3)	1 (0.5)	p<0.001
20-24	380835 (15.3)	12 (6.5)	
25-29	780008 (31.4)	45 (24.2)	
30-34	709725 (28.6)	47 (25.3)	
35-39	378431 (15.2)	46 (24.7)	
40-44	141177 (5.7)	25 (13.4)	
45-49	44245 (1.8)	4 (2.2)	
>50	17388 (0.7)	6 (3.2)	
Total	2483591 (100.0)	186 (100.0)	
Mothers			
Age group			
=<19	143372 (5.7)	2 (1.0)	

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20-24	649775 (25.9)	27 (13.0)	p<0.001
25-29	857129 (34.1)	58 (29.7)	
30-34	589846 (23.5)	63 (32.3)	
35-39	228408 (9.1)	43 (22.1)	
>=40	42724 (1.7)	2 (1.0)	
Total	2511154 (100.0)	195 (100.0)	
Parity			
0	1038409 (41.4)	105 (53.8)	p<0.001
1	873136 (34.8)	72 (36.9)	
2	409303 (16.3)	17 (8.7)	
3	126986 (5.1)	1 (0.5)	
>=4	63368 (2.5)	0 (0.0)	
Total	2511202 (100.0)	195 (100.0)	
Multiple pregnancies			
Births from twin pregnancies	66836 (2.7)	8 (4.7)	n.s.
Total	2511202 (100.0)	195 (100.0)	

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Table 2.

Complications during pregnancy and delivery when comparing pregnancies after transplant in mothers with pregnancies in the general population, Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mothers and fathers age, parity and plurality.

	195 births after transplant n (%)	2511202 births in the general population n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Preeclampsia	42 (21.6)	77457 (3.1)	8.7 (6.2-12.2)	6.5 (5.0-9.1)
Preterm delivery (<37 weeks)	66 (34.0)	154027 (6.1)	7.9 (5.8-10.6)	7.1 (5.3-9.6)
Preterm delivery (<34 weeks)	28 (14.4)	51575 (2.1)	8.0 (5.4-12.0)	7.5 (5.0-11.1)
Induction of labor	46 (23.7)	387823 (15.4)	1.7 (1.2-2.4)	1.6 (1.2-2.2)
Operative vaginal delivery	10 (5.2)	170490 (6.8)	1.3 (0.7-2.5)	0.5 (0.2-0.9)
Caesarean section	121 (62.4)	257660 (10.3)	15.0 (10.8-19.4)	9.7 (7.2-13.0)
Apgar<7 at 5 min	2 (1.1)	26779 (1.5)	0.7 (0.2-2.9)	0.6 (0.2-2.6)

*Only 185 transplanted patients and 1798146 non-transplanted patients were considered in the analysis of this variable

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Table 3.

Perinatal complications and malformations when pregnancies after transplant were compared to pregnancies in the general population, Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mother's age and parity. Low birth weight was also adjusted for gestational age.

	195 births after transplant n (%)	2511202 births in the general population n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Small for Gestational Age (<10 percentile)*	42 (22.7)	269944 (11.3)	2.3 (1.6-3.2)	2.5 (1.7-3.5)
Large for Gestational Age (>90 percentile)*	4 (2.2)	227807 (9.6)	0.2 (0.1-0.6)	0.21 (0.1-0.6)
Low Birth Weight	57 (29.4)	131130 (5.2)	7.5 (5.5-10.3)	2.7 (1.7-4.1)
Stillbirth	5 (2.6)	26081 (1.0)	2.5 (1.0-6.1)	2.9 (1.2-7.0)
Perinatal mortality	7 (3.6)	36554 (1.5)	2.5 (1.2-5.4)	3.2 (1.5-6.7)
Major malformations	8 (4.1)	52359 (2.1)	2.0 (0.99-4.1)	1.6 (0.8-3.3)

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Table 4.

Complications during pregnancy and delivery and perinatal outcomes when comparing pregnancies before and after transplantation in the same patient. Norway 1969-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for year of birth, mothers and fathers age, parity and plurality. Low birth weight was also adjusted for gestational age.

	26 births after transplant n (%)	26 births before transplant n (%)	Crude OR (95% CI)
Preeclampsia	5 (19.2)	7 (26.9)	0.6 (0.2-2.4)
Preterm delivery (<37 weeks)	10 (38.5)	10 (38.5)	1 (0.3-3.1)
Induction of labor	5 (19.2)	11 (42.3)	0.3 (0.1-1.1)
Operative vaginal delivery	1 (3.8)	3 (11.5)	0.3 (0.0-3.2)
Caesarean section	19 (73.1)	12 (46.2)	3.2 (1.0-10.1)
Small for Gestational Age (<10 percentile)	6 (23.1)	8 (30.8)	0.6 (0.2-2.2)
Large for Gestational Age (>90 percentile)	0	0	
Low Birth Weight	11 (42.3)	13 (50)	0.7 (0.2-2.2)

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Stillbirth	1 (3.8)	5 (19.2)	0.2 (0.0-1.6)
Perinatal mortality	1 (3.8)	5 (19.2)	0.2 (0.0-1.6)
Major malformations	1 (3.8)	1 (3.8)	1 (0.1-16.9)

*Only the last birth before transplantation and the first birth after transplantation were considered in the analysis.

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Figure Legend

Figure 1

Number of pregnancies after solid organ transplantation by time period, Medical Birth Registry of Norway, 1969 to 2009.

Figure 2

Proportion of preterm delivery (PTD) in pregnancies after kidney transplantation by time period, Medical Birth Registry of Norway, 1969-2009.

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Figure 1

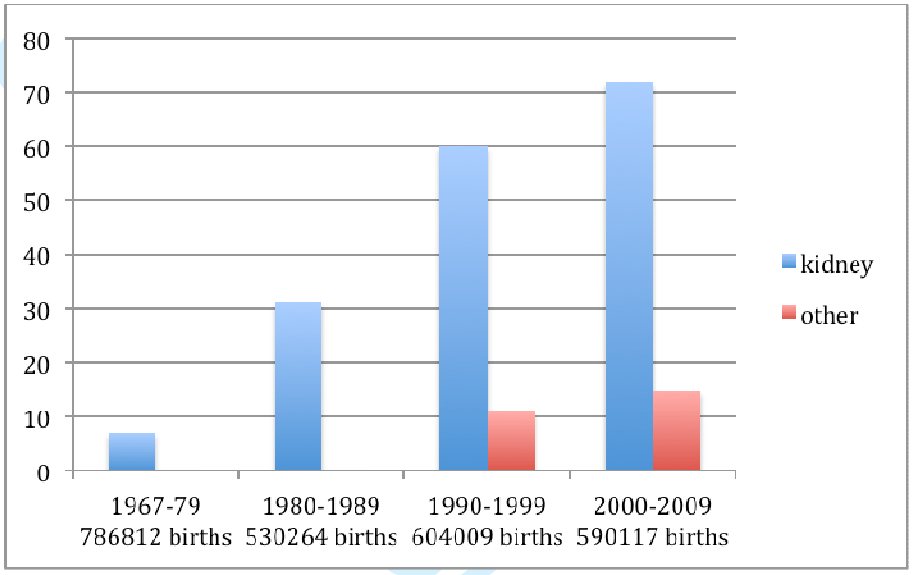

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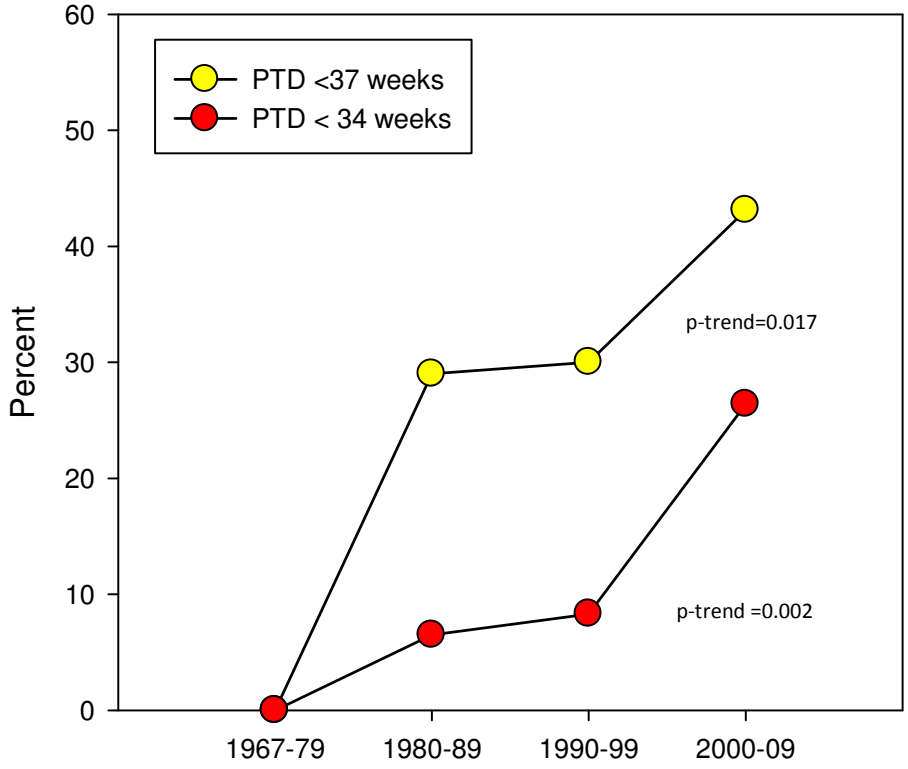
Figure 2



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Supplementary Table 1.

Complications during pregnancy and delivery when comparing pregnancies after and before transplantation, Norway 1967-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for confounders as given.

	2260 births before transplant n (%)	195 births after transplant n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Preeclampsia	244 (10.8)	42 (21.6)	2.3 (1.6-3.3)	0.97 (0.63-1.49)
Prelabor rupture of membranes	52 (2.3)	12 (6.2)	2.8 (1.5-5.3)	1.47 (0.67-3.20)
Bleeding during pregnancy	127 (5.6)	32 (16.5)	3.3 (2.2-5.0)	1.27 (0.77-2.10)
Preterm delivery (<37 weeks)	318 (14.1)	66 (34.0)	3.1 (2.3-4.3)	1.32 (0.91-1.94)
Induction of labor	489 (21.6)	46 (23.7)	1.1 (0.8-1.6)	0.73 (0.49-1.07)
Operative vaginal delivery	115 (5.1)	10 (5.2)	1.0 (0.5-2.0)	0.57 (0.27-1.21)
Caesarean section	342 (15.1)	121 (62.4)	9.3 (6.8-12.7)	2.81 (1.95-4.03)

All OR are adjusted for year of birth, mother's age and parity.

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Supplementary Table 2.

Perinatal complications and malformations when mothers were transplanted, before and after transplantation, Norway 1967-2009. Odds ratios (OR) with 95% confidence intervals (CI) were obtained using logistic regression analysis and adjusted for confounders as given.

	2260 births before transplant n (%)	195 births after transplant n (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Small for Gestational Age (<10 percentile)*	430 (20.4)	42 (22.7)	1.1 (0.8-1.6)	1.16 (0.76-1.76)
Large for Gestational Age (>90 percentile)*	175 (8.3)	4 (2.2)	0.2 (0.1-0.7)	0.26 (0.09-0.74)
Low Birth Weight	325 (14.4)	57 (29.4)	2.5 (1.8-3.5)	1.73 (0.45-1.20)
Apgar <7 at 5 min*	40 (4.1)	2 (1.1)	0.3 (0.1-1.1)	0.12 (0.03-0.53)
Stillbirth	75 (3.3)	5 (2.6)	0.8 (0.3-1.9)	0.53 (0.19-1.47)
Perinatal mortality	106 (4.7)	7 (3.6)	0.8 (0.4-1.7)	0.61 (0.26-1.44)
Infant death	52 (2.3)	2 (1.0)	0.4 (0.1-1.8)	0.37 (0.08-1.71)
Major malformations	57 (2.5)	8 (4.1)	1.7 (0.8-3.5)	0.82 (0.34-1.95)

All OR are adjusted for year of birth, mother's age and parity. Low birth weight is also adjusted for gestational age.

First clinical uterus transplantation trial: a six-month report

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Objective: To report the 6-month results of the first clinical uterus transplantation (UTx) trial. This type of transplantation may become a treatment of absolute uterine-factor infertility (AUFI).
Design: Prospective observational study.
Setting: University hospital.
Patient(s): Nine AUFI women and their live uterine donors, the majority being mothers.
Intervention(s): Live-donor UTx and low-dose induction immunosuppression.
Main Outcome Measure(s): Data from preoperative investigations, surgery and follow-up for 6 months.
Result(s): Durations of donor and recipient surgery ranged from 10 to 13 hours and from 4 to 6 hours, respectively. No immediate perioperative complications occurred in any of the recipients. After 6 months, seven uteri remained viable with regular menses. Mild rejection episodes occurred in four of these patients. These rejection episodes were effectively reversed by corticosteroid boluses. The two graft losses were because of acute bilateral thrombotic uterine artery occlusions and persistent intrauterine infection.
Conclusion(s): The results demonstrate the feasibility of live-donor UTx with a low-dose immunosuppressive protocol.
Clinical trial registration number: NCT01844362. (Fertil Steril® 2014;101:1228–36. ©2014 by American Society for Reproductive Medicine.)
Key Words: Infertility, human, transplantation, uterus

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Uterine-factor infertility is caused by absence or dysfunction of the uterus. This untreatable condition, which is either congenital or acquired, affects 1 in 500 fertile-age women (1–4), corresponding to ~200,000 women in Europe. Surrogacy or adoption may be satisfactory options for many women with uterine-factor infertility but may be unacceptable for others owing to ethical, legal, or religious concerns (5). In Sweden, surrogacy is not legally approved.

We have examined uterus transplantation (UTx) as a possible infertility treatment. During the past decade, we have stepwise studied and optimized the procedure in animals, with the aim of clinical implementation (6–8). The ethics around UTx are unquestionably complex and have been the focus of several publications (9, 10). A controlled trial on human UTx would shed some light on important questions, such as medical and psychologic risks and benefits. Unlike any other transplantation currently performed, UTx is ephemeral; it is not intended for

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lifelong duration. The graft is removed after one or two healthy babies have been born, to limit the immunosuppression period.

Since the first birth in a woman transplanted with a solid organ half a century ago (11), more than 15,000 babies have been born to transplanted and immunosuppressed mothers, with no reported increased risk of fetal malformation (12). This indicates that human UTx and accompanying immunosuppression are compatible with normal pregnancy and progeny.

There have been two previous UTx attempts, both by teams with no preceding research records in the field. The first case, in 2000, resulted in graft failure with hysterectomy after 3 months (13). The reason for failure was not clear, but the authors stated that the likely cause was prolapse of the organ due to poor pelvic fixation, which led to compression of blood vessels and vascular thrombosis. The second case, in 2011, involved a uterine graft from a deceased female multiorgan donor (14). This case has also presented two pregnancies but with early miscarriage (15).

Our research on UTx started more than 10 years ago (16) and we have, by a scientific step-by-step approach, examined and optimized the UTx procedure. This animal-based research has been carried out in rodents, large domestic animals, and a nonhuman primate (7, 8). In 2012, we were granted ethical permission to conduct a clinical trial on human UTx at Sahlgrenska University hospital with uteri from live donors. At the time of writing, the entire cohort of nine recipients, with their donors, has been monitored for 6 months after transplantation. In the present paper, we report the perioperative and 6-month postoperative outcome of the nine donor-recipient pairs entering the UTx trial.

METHODS

Patients and Pretransplantation Investigations

The Regional Human Ethics Committee approved a study of up to ten live-donation UTx procedures. We performed two initial cases in September 2012, and after an extended observation period of these cases we later decided to perform a prospective observational study, with the trial being registered at ClinicalTrials.gov (registration no. NCT01844362). A preliminary screening process of 30 prospective recipients ended in selection of ten suitable women. The reasons for exclusion were medical or psychologic risk factors in recipients or planned donors. All of the recipients and their partners had been thoroughly counseled about their national and international options to gain parenthood through adoption or surrogacy. Multiple visits to psychologists and independent doctors (Table 1) assured that they were fully aware of the research nature of the trial. In vitro fertilization was performed to exclude any sterility factor related to fertilization failure and to cryopreserve embryos for transfer more than 12 months after transplantation, according to international transplantation recommendations (17). An independent Transplantation Board finally assessed each donor-recipient pair and excluded one pair because the recipient was found to have bilateral pelvic kidneys. Written informed consents were obtained from all donors, recipients, and their partners. All investigation procedures, surgeries, and follow-up visits were at the Sahlgrenska University hospital.

TABLE 1

Preoperative medical investigations of recipients and donors.

Radiology	MRI (abdominal and pelvic)
	Chest x-ray
	Vaginal ultrasound scan
Clinical	Electrocardiography (ECG)
	Exercise ECG[a]
	Pap smear
Blood chemistry	
Liver function	Alanine transaminase (ALT)
	Aspartate transaminase (AST)
	Alkaline phosphatase (ALP)
	Albumin
	Total protein
	Bilirubin
Kidney function	Creatinine
	Urea
	Electrolytes
	Dissolved salts
General	Hemoglobin
	White blood cells (WBC)
	Prothrombin time (PT)
	Activated partial thromboplastin time (APTT)
	Total particle concentration (TPC)
	C-Reactive protein (CRP)
Microbiology	Cytomegalovirus (CMV)
	Epstein-Barr virus (EBV)
	Human immunodeficiency virus (HIV)
	Hepatitis A, B, C
	Chlamydia
	Human papilloma virus (HPV)
	Gonorrhea
	Syphilis
Assessment by specialist in	Gynecology
	Transplantation surgery
	Psychology
	Clinical immunology
	Anesthesiology
	Internal medicine
	Radiology

Note: MRI = magnetic resonance imaging.
[a] Donors only.

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The characteristics of recipients and donors are presented in Table 2. Five of the donors were mothers of recipients, and four of these were postmenopausal. Eight patients had the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome with congenital absence of the uterus and vagina (18), and one recipient had undergone radical hysterectomy for cervical cancer. All recipients had been in a steady relationship with their partners for ≥ 3 years.

Immunology and Microbiology

Donors' and recipients' human leukocyte antigen (HLA) A, B, C, DRβ1, and DQβ1 loci were typed with the use of polymerase chain reaction (PCR); reverse sequence-specific oligonucleotides (LABtype; One Lambda) and their degrees of HLA mismatch (at the A, B, and DRβ1 loci) were determined. HLA antibodies were detected with the use of the Luminex-based LABscreen panel reactive antibodies assay (One Lambda). A mean fluorescence intensity (MFI) value >1,000

TABLE 2

Subjects' characteristics.

	Recipients	Donors	Partners
n	9	9	9
Age (y)	31.5 ± 3.9	53.0 ± 7.0	34.3 ± 4.0
BMI	22.4 ± 1.5	25.6 ± 4.2	
Previous smoking, n (%)	3 (33)	4 (44)	
Previous abdominal surgery, n (%)			
Laparotomy	2 (22)	3 (33)	
Laparoscopy	6 (67)		
Urogynecologic characteristics, n (%)			
Kidney malformation	4 (44)		
Single kidney	3 (33)		
Unilateral pelvic kidney	1 (11)		
Cause of absent uterus, n (%)			
MRKH	8 (89)		
Cervical cancer	1 (11)		
Type of vagina, n (%)			
Normal	1 (11)	9 (100)	
Self-dilated	3 (33)		
Therapeutically dilated	1 (11)		
Skin	4 (44)		
Pregnancies		3.3 ± 1.3	
Live births		3.0 ± 0.9	
Deliveries, n (%)			
Vaginal		25 (93)	
Cesarean section		2 (7)	
Menopausal state, n (%)			
Premenopausal		4 (44)	
Postmenopausal			
<5 y		2 (22)	
>5 y		3 (33)	

Note: Plus-minus values are mean ± SD. BMI = body mass index; MRKH = Mayer-Rokitansky-Küster-Hauser syndrome.

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above the negative control was considered to be positive. Both standard complement-dependent cytotoxicity and flow-cytometric crossmatch tests with the use of T and B lymphocytes were performed. Flow cytometry was performed on a FACScalibur cytometer using a 1,024 linear scale and the Cellquest Pro software (BD Biosciences). They were also tested for relevant viral and bacterial infections (Table 1) with standard techniques. The partners were tested for HIV, hepatitis B and C, and syphilis.

Anesthesia and Postoperative Medication

Anesthesia was similar in donors and recipients. Before the start of general anesthesia, a mixture of 10 mg bupivacaine (Marcain Spinal; AstraZeneca) and 0.1 mg morphine (Morfin Special; Biophausia) was given intrathecally at the L3–4 or L4–5 level. Subsequent induction of anesthesia started with intravenous (IV) infusions of remifentanil (Ultiva; GlaxoSmithKline) at a rate of 0.25 mg/kg/min, together with a bolus (2–3 mg/kg body weight [bw]) of propofol (Fresenius), followed by 40–50 mg rocuronium bromide (Esmeron; MSD) intravenously. The patient was then intubated and maintenance anesthesia administered with sevoflurane (Sevofluran Baxter; Baxter) and remifentanil infusion (Ultiva). Anesthesia depth was monitored and adjusted, with a target minimal alveolar concentration of 0.5–0.7, and further monitored by maintaining electroencephalographic and frontal electromyographic signal activity (Entropy; GE Healthcare) at a level of ~25%–35%.

Up to 3 L Ringer acetate and 0.5 L dextran-70 (Macrodex; Meda) were given perioperatively. To maintain fluid balance, 500 mL hydroxyethyl starch (Tetraspan; Braun,) or 20 g albumin (Albumin Baxter; Baxter Medical) was administered as needed. Dopamine (Giludop; Abcur) infusion was used to keep mean arterial pressure at a level of >65 mm Hg. An autologous blood recovery system (Cell Saver; Haemonetics) was available during surgeries. The surgical duration for each patient was defined as the period from the first skin incision to completed skin closure.

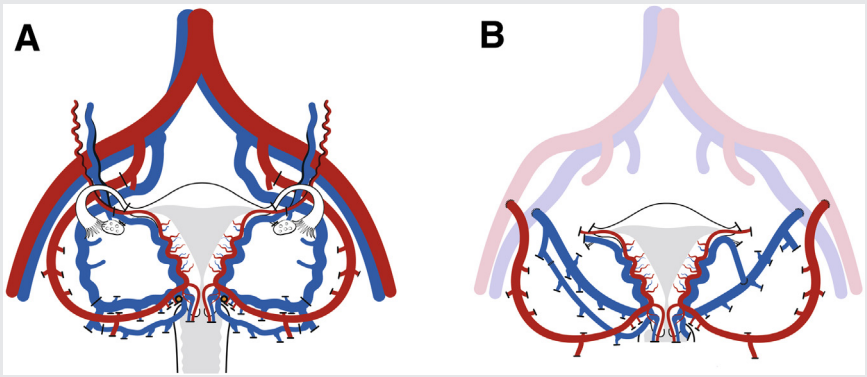
To reduce postoperative nausea, 4–8 mg ondansetron (Ondansetron; Braun), 4 mg betamethasone (Betapred; Sobi), and 0.5–1 mg droperidol (Dridol; Postrakan) were administered intravenously at the end of surgery. Paracetamol (Perfalgan; Bristol-Myers Squibb), parecoxid (Dynastat; Pfizer), tramadol (Tradolan; Nordic Drugs), and morphine (Morfin Meda; Meda) were used as initial postoperative pain relief.

Antibiotics (4 g; Piperacillin/Tazobactam; Farmaplus) were given once preoperatively and three times daily for 1 day in donors and 3 days in recipients. Thrombosis prophylaxis for donors was with dalteparin (5,000 IU; Fragmin; Pfizer) during postoperative days (PODs) 1–21. Thrombosis prophylaxis for recipients was with acetylsalicylic acid (75 mg; Trombyl; Pfizer) once daily throughout the 6-month follow-up period and with dalteparin (5,000 IU; Fragmin) during PODs 1–42.

Donor Surgery

Donor surgery involved a midline incision from the pubic bone to the umbilicus, followed by isolation of the uterus with long vascular pedicles consisting of the bilateral uterine arteries and veins up to and including parts of the internal iliac vessels (Fig. 1A). Substantial parts of the round ligaments and the sacrouterine ligaments, as well as an extensive sheet of the bladder peritoneum, were preserved on the graft side to enable stable fixation of the uterus in the recipient. Bilateral salpingectomy was performed, preserving the uterine branch of the utero-ovarian vein (Fig. 1A). Dissections on the pelvic sidewalls included dissection of the ureters from their passages over the iliac vessel bifurcations distally to their inlets into the bladder. This included meticulous dissection of the uterine veins and uterine arteries from their firm attachments to the ureters. When the ureters had been completely mobilized from the cervix and uterine vessels, vascular dissection aimed at mobilizing the internal iliac arteries and veins started from the bifurcations of the internal and external iliac vessels and proceeded distally (Fig. 1A). This dissection included severance of multiple major vascular branches. The vagina was transected 10–15 mm caudal to the vaginal fornix, and the uterus was eventually attached to the donor by only its two arterial and two venous vascular pedicles. The major feeding arteries and veins were then clamped and severed, before the uterus was removed from the pelvis to a back-table setting.

FIGURE 1



Overview of transections, ligations, and anastomosis lines at uterus transplantation. (A) Schematic drawing of the arteries (*red*) and veins (*blue*) connected to the uterus. Transection lines are indicated by *black lines*. (B) The uterus in place in the pelvis of the recipient with bilateral end-to-side anastomoses on the recipient's external iliac vessels.

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The clamping points of the donor's internal iliac vessels were closed with continuous polypropylene (6-0) sutures and the abdomen was closed in standard three-layered manner. A suprapubic bladder catheter was inserted to avoid urinary retention secondary to the extensive dissection around the bladder. The suprapubic catheter was removed on day 4 or 5 after surgery, when the residual urinary volume had decreased to <150 mL. Anesthesia was discontinued and the donor was brought to the postoperative recovery unit.

Graft Preparation

The uterus was quickly brought to the back-table and initially flushed with 10 mL heparinized saline solution through each artery, followed by flushing for 10–20 minutes with cold preservation solution (Custodiol HTK-solution; Nordmedica), until the organ was blanched and the venous effluent was clear. The graft was kept on ice until transplanted into the recipient. In some patients, back-table preparation involved anastomosing (7-0 polypropylene) one ovarian vein end-to-side to the uterine vein (Fig. 1B) to achieve greater venous drainage, whereas this branch of the ovarian-uterine vein was subsequently directly anastomosed to the external iliac vein of the recipient in others (see below). Organ ischemia was divided into three distinct periods. The first warm ischemia period was from cross-clamping at organ retrieval to commencement of cold flushing. The cold ischemia period was from initiation of organ flushing to as long as the organ was kept on ice. The second warm ischemia period was the time from removal of the organ from ice to blood reperfusion. Because the first warm ischemia period was <2 minutes in all cases, this period was added to the longer second warm ischemia period and data on warm ischemia are presented as the sum of these periods.

Recipient Surgery

To synchronize donor and recipient surgeries, thereby avoiding a long cold ischemia period for the uterine graft, a second team of surgeons started the preparatory surgery of the recipient in an adjacent operating room well before the anticipated procurement of the organ. This laparotomy also was performed through a subumbilical midline incision. First, the vaginal vault was dissected free from the bladder and the rectum. In the eight MRKH patients, the uterine rudiment had to be cleaved to reach the top of the vagina. Sutures (1-0 polypropylene) for subsequent organ fixation were attached to the round ligaments, the sacrouterine ligaments, and the two lateralized parts of the uterine rudiment (MRKH patients) or the paravaginal connective tissue (cervical cancer patient). The surgery was then directed toward preparation of the external iliac vessels for subsequent anastomosis. The external iliac artery and vein were bilaterally separated from each other and from adjacent tissue to a distance of ~60 mm.

The uterine graft was then brought, still on ice, into the recipient's operating room. It was placed in its normal position in the pelvis and bilateral end-to-side vascular anastomoses were performed between the graft vessels and the external iliac vessels with the use of continuous 7-0 (arterial anastomosis) or 8-0 (venous anastomosis) polypropylene sutures. After each venous anastomosis had been sutured, the vascular clamp over the external iliac vein was opened. In some cases, single sutures were used to seal any leakage from the anastomosis line. In six patients, the uterine branch of the ovarian-uterine vein was used and anastomosed to the ipsilateral uterine vein or prepared for direct anastomosis to the external iliac vein (Fig. 1B). Mannitol (30 g; Mannitol Baxter Viaflo; Baxter Medical) was given as an IV bolus just before the arterial clamps were removed, and systolic blood pressure was carefully monitored and maintained at >100 mm Hg. After completed vascular anastomosis surgery, adequate blood flow through the uterine arteries of the vessels was verified and quantified with a vascular Doppler probe (Vascular TTFM Probe; Medistim) placed around the uterine artery.

The recipient's vaginal vault was then opened by a longitudinal incision of ~40 mm. The vaginal rim of the graft was anastomosed to the top of the recipient's vagina with a

continuous absorbable 2-0 suture. The uterus was fixed in its pelvic location by attaching the uterine ligaments to their pelvic counterparts with the previously placed fixation sutures, and by overlaying the extensive bladder peritoneum of the graft over the recipient's bladder fundus. A Doppler probe with a silicon cuff (Cook-Schwartz Doppler probe; Cook Medical) was then placed around one uterine artery to ascertain that pulsatile blood flow was maintained during the initial 72-hour postoperative period, with the signal transduced from a 20-MHz crystal on the tip of a thin cable through the midline incision. The cable, with its crystal, was then gently pulled out from its intra-abdominal position, leaving the silicon cuff in situ. The surgery and anesthesia of the recipients were terminated identically to the donors.

Immunosuppression and Long-Term Medication

The recipients followed a standardized protocol of induction and maintenance immunosuppression. Preoperatively, the recipient had received 1g mycophenolate mofetil (MMF; Cellcept; Roche). During transplantation, before reperfusion, she was given 500 mg methylprednisolone (Solu-Medrol; Pfizer) and antithymocyte antibodies, to deplete T lymphocytes, either thymoglobulin (IV, 2.5 mg/kg bw; Genzyme Aps) or antithymocyte globulin (ATG; IV, 5 mg/kg bw; ATG-Fresenius; Fresenius), with identical doses of each drug repeated 12 hours later.

Maintenance immunosuppressive therapy was continued with tacrolimus (Prograf,-Astellas Pharma) twice daily (adjusted to trough levels of 10–15 ng/mL during weeks 1–5 and 5–10 ng/mL during week 6 and thereafter) and MMF twice daily with MMF area under the curve trough levels of 40–60 mg·h/L. Oral glucocorticosteroids (Prednisolon; Pfizer) were administered once daily on the day of surgery and during the first 4 postoperative days.

Antiviral prophylaxis consisted of a daily oral dose (450 mg) of valganciclovir (Valcyte; Roche), which was given for 3 or 6 months, depending on cytomegalovirus status (3 months when both donor and recipient were positive; 6 months when donor was positive and recipient negative). When both donor and recipient were negative, prophylaxis was not given.

Follow-up

Surgical complications were registered with the use of Clavien-Dindo classification (19). The recipients were monitored by clinical examination twice weekly during the 1st month, once weekly during months 2–3, and thereafter every other week. The uterus and endometrium were examined with vaginal and abdominal two-dimensional ultrasonography (Flex Focus 400; BK Medical). Color Doppler ultrasound was used to assess that blood flow was maintained through the uterine arteries. Clinical examination included visual inspection of the uterine cervix as well as cervical cultures and biopsies. The biopsies were obtained at predetermined time points (1, 2, and 4 weeks, and monthly thereafter) as well as on suspicion of graft rejection based on clinical examination (discolored cervix, abnormal vaginal discharge, enlarged uterus, fever, abdominal pain). Drug levels (tacrolimus, MMF) were monitored by standard assays, and routine blood tests were performed to assess liver and kidney function and to detect infection.

Rejection was diagnosed based on histopathologic examination of cervical biopsies and graded according to the proposed rejection classification for the primate uterus (6).

Statistics

Values are presented as individual values and as mean ± SD.

RESULTS

Matching and Immunology of Patients

Seven donor/recipient pairs were ABO identical, and two were compatible. Both cytotoxic and flow-cytometric crossmatch tests were negative in all patients, and no patients had HLA antibodies. The degree of HLA mismatch among the donor and recipient pairs varied from 1/0 to 3/2 (Table 3).

Surgery

Surgical parameters are presented in Table 3. Donor surgery lasted 10–13 hours. Recipients 1 and 2 underwent prolonged anesthesia because recipient and donor surgeries were improperly synchronized, so that 9- to 10-hour waiting periods occurred before graft anastomosis. Blood transfusion was not required during any surgery, although volumes <0.6 L were returned by the autologous blood recovery system in seven cases. Cold ischemia periods lasted 1 to 2 hours. Major interindividual variation in dominant uterine artery blood flow, recorded by Doppler, was observed (Table 3).

Postoperative Period and Complications: Donors

The initial postoperative hospital stay of the donors was 6 days (Table 3), with no need for intensive care. One grade IIIb surgical complication occurred. Donor 2 presented on POD 16 with watery vaginal discharge due to a ureterovaginal fistula. A pyelostomy catheter was inserted and her ureter was reimplanted on POD 134, after which recovery was uneventful.

Postoperative Period and Complications: Recipients

The first two recipients experienced nausea and dyspnea on POD 1, with chest x-rays revealing pleural fluid (grade I complications). They were observed at an intermediary care unit until the symptoms ceased and the pleural fluid resorbed spontaneously (POD 2–3). No other recipient experienced these symptoms. A grade II complication (blood transfusion) occurred in one recipient on POD 2 (Table 3), and a retroperitoneal hematoma was visualized on computerized tomographic (CT) scan.

Two recipients developed complications necessitating surgical removal of the graft (grade IIIb complications). Recipient 2 was readmitted on POD 33 with abdominal pain, fever, and vaginal discharge. Gynecologic examination revealed signs of cervical/uterine infection, confirmed by positive

TABLE 3

Surgical, anesthesiologic, and hospitalization parameters.

Pair	Age (y)	Donors' relation to recipients	HLA mismatch	Cytomegalovirus Ig	Epstein-Barr virus Ig	Duration: Anesthesia	Duration: Surgery	Duration: Back-table Preparation	Duration: Anastomoses Veins	Duration: Anastomoses Arteries	Duration: Ischemia Warm	Duration: Ischemia Cold	Arterial blood flow (mL/min)[a]	Blood loss (L)	Intraop. autoblood (L)	Blood transfusion (L)[b]	Highest grade of complication[c]	Postop. hospitalization (d)
Recipient 1	33	Mother	2/0	pos	pos	15 h 0 min	4 h 10 min	1 h 30 min	39 min	35 min	1 h 18 min	1 h 30 min	63	0.4	0	0	Grade II	8
Donor 1	52			neg	pos	12 h 4 min	10 h 54 min							0.3	0	0		6
Recipient 2	38	Mother	2/1	neg	neg	13 h 57 min	4 h 17 min	1 h 47 min	43 min	29 min	1 h 38 min	1 h 47 min	50	1.6	0.49	0	Grade IIIb	9
Donor 2	58			pos	pos	13 h 46 min	12 h 37 min							2.4	0.49	0	Grade IIIb	6
Recipient 3	28	Mother's sister	3/1	pos	pos	10 h 50 min	4 h 50 min	1 h 4 min	31 min	25 min	1 h 34 min	1 h 4 min	50	0.8	0.2	0		6
Donor 3	54			neg	pos	13 h 37 min	12 h 53 min							0.8	0	0		6
Recipient 4	27	Mother	2/1	neg	pos	6 h 5 min	5 h 4 min	0 h 57 min	32 min	30 min	1 h 17 min	0 h 57 min	43	0.25	0	0		6
Donor 4	50			neg	neg	11 h 11 min	10 h 34 min							0.6	0	0		6
Recipient 5	35	Family friend	3/2	pos	pos	5 h 45 min	4 h 55 min	1 h 6 min	32 min	30 min	1 h 13 min	1 h 6 min	40	0.75	0	1.2	Grade II	6
Donor 5	61			pos	pos	11 h 6 min	10 h 17 min							0.6	0	0		6
Recipient 6	27	Mother	1/1	neg	pos	8 h 17 min	4 h 30 min	2 h 0 min	60 min	23 min	1 h 24 min	2 h 0 min	75	0.7	0.18	0		3
Donor 6	53			pos	pos	11 h 50 min	10 h 52 min							0.7	0.11	0		6
Recipient 7	28	Mother	1/0	pos	pos	6 h 35 min	4 h 44 min	0 h 54 min	20 min	30 min	1 h 15 min	0 h 54 min	30	0.3	0	0	Grade II	7
Donor 7	50			pos	pos	11 h 35 min	10 h 17 min							0.4	0	0		6
Recipient 8	33	Sister	1/1	pos	pos	7 h 53 min	5 h 56 min	0 h 56 min	25 min	24 min	1 h 14 min	0 h 56 min	30	0.75	0.2	0	Grade II	8
Donor 8	37			pos	pos	11 h 55 min	11 h 23 min							0.4	0	0		6
Recipient 9	35	Mother-in-law	3/2	neg	neg	8 h 14 min	4 h 31 min	1 h 28 min	42 min	47 min	1 h 32 min	1 h 28 min	10	0.5	0	0	Grade IIIb	7
Donor 9	62			pos	pos	14 h 5 min	13 h 8 min							2.1	0.52	0		6
All recipients	31.5 ± 3.9					9 h 4 min ± 3 h 14 min	4 h 46 min ± 30 min	1 h 18 min ± 23 min	36 ± 11 min	30 ± 6.9 min	1 h 23 min ± 9 min	1 h 18 min ± 23 min	44 ± 18	0.67 ± 0.38	0.12 ± 0.15	0.13 ± 0.36		6.7 ± 1.6
All donors	53.0 ± 7					12 h 13 min ± 60 min	11 h 37 min ± 1 h 5 min							0.92 ± 0.73	0.12 ± 0.21	0 ± 0		6.0 ± 0

Note: Plus-minus values are mean ± SD.
[a] Peak value of the dominant artery.
[b] Packed red cells.
[c] According to the Clavien-Dindo classification of surgical complications (17).

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Enterococcus faecalis culture. After 4 days of intravenous antibiotics, the symptoms resolved; the recipient was discharged on POD 38 with oral antibiotics. She was kept on oral antibiotics owing to persisting *Enterococci* in cervical cultures and a modest increase in C-reactive protein (CRP), except during one febrile episode (POD 78–83) when IV antibiotics were temporarily reintroduced. She was readmitted on POD 98 owing to aggravated symptoms, and an intrauterine abscess measuring 3×4 cm was seen on CT scan. Despite two surgical drainage attempts, the abscess persisted and the patient developed initial signs of septicemia. Hysterectomy was performed on POD 105. Morphologic examination revealed extensive areas of necrosis, with some neutrophil-dominated inflammation, but no signs of rejection. Subsequent recovery was uneventful.

Recipient 9 had sudden cessation of the uterine artery Doppler signal on the morning of POD 3, and gynecologic examination revealed a blood-congested cervix. Acute laparotomy revealed a congested uterus and uterine arteries without palpable pulses. The entire graft was removed and morphologic examination revealed focal necrosis and moderate ischemic myometrial damage, but no signs of rejection. Occluding thrombi were found in both major arteries and veins. The patient was discharged from the hospital 5 days after hysterectomy, with frequent initial follow-up visits and contacts with the team's doctors and psychologist. At 6-month follow-up, she was back at work and had experienced no physical sequelae.

Rejection and Uterine and Renal Function

Three (numbers 1, 5, and 7) of the seven recipients with viable uteri throughout the observation period, suffered single episodes of mild rejection during the 1st month. One recipient (number 8) had two episodes of mild rejection (month 1 and month 3). Morphologic analysis of the cervical tissue showed mild infiltration of lymphocytes and neutrophils, mainly in the basal squamous epithelium of the ectocervix. All rejection episodes (grade II complications) were successfully treated with corticosteroids for 7–10 days. Spontaneous menstruations resumed within 2 months in the seven patients with regular menstrual patterns (interval 27–32 days) and with a mean maximum endometrial thickness of 14 ± 3 mm.

Preoperative creatinine levels, mirroring renal function, were 7.35 ± 9.1 μmol/L, and a reversible increase (maximal increase 37.1 ± 20.6 μmol/L) occurred during the initial 1–2 months of immunosuppression.

DISCUSSION

This study represents the first clinical trial of human UTx, after two solitary human UTx attempts in 2000 (13) and 2011 (14). In contrast to those single cases, which were not preceded by any internal UTx research, our team has been involved in animal-based UTx research for several years (6–8). In the rodent models, we have studied rejection patterns in a uterine allograft, immunosuppression to avoid uterine graft rejection, and, importantly, pregnancies and offspring after UTx. Large animal models, including primates, have mostly been used to optimize the surgery at UTx and to evaluate suitable combinations of immunosuppressants. Our introduction of human UTx to the clinical arena complies with the recently released IDEAL recommendations for clinical introduction of major surgical procedures (20).

Uterus transplantation, as a non–life-saving transplantation and a technically complicated infertility treatment, raises several ethical questions (21–23) regarding priorities in the health care sector, as well as the risks and benefits of the procedure. A clinical trial such as ours generates important data on the risks and benefits, which will also be advantageous in an ethical analysis of UTx.

We recovered the uterine grafts from live donors. Live donation of organs is most common in renal transplantation; ~40% of transplanted kidneys are from live donors in the United States (24). Because our prior UTx research in sheep (25) and baboons (6, 26) followed the live donor concept, our team was well prepared for that particular surgical procedure. All donors had undergone at least one normal pregnancy. In the recent UTx case in Turkey (14), the uterus was obtained from a nulliparous deceased donor and the local team assembled on short notice for surgery. The recipient has subsequently undergone two pregnancies, with early miscarriages occurring both times (15). The cause of these pregnancy failures may be related to intrinsic factors in the particular uterus or to the triple immunosuppression still administered. As recently outlined by Donnez (27), there may exist specific factors of a transplanted uterus, such as decreased vascular plasticity, placentation defects, and loss of innervation, that may negatively affect the pregnancy potential in a uterine allograft.

Our team, with surgeons from three continents, had to plan the exact dates of the surgeries well in advance. Furthermore, our hospital board required that all surgeries be done during weekends in operating rooms that were usually closed on weekends. Deceased-donor UTx would not be a realistic option under these conditions.

Before the first UTx procedure, we had anticipated considerably easier and faster retrieval surgery than was actually the case, because uterus retrieval in our baboon UTx studies took ~2.5 hours (6, 26). Therefore, and based on our feasibility study of uterine vessel dissection at radical hysterectomy, we had predicted a duration of 3–4 hours (28). In that study, the median surgery duration was ~5 hours, when uterine vessel dissections were included in the hysterectomy and lymph node dissection procedure. The most time-consuming surgical step in retrieval surgery in the present study was ureter and uterine vessel dissection, which took ~4–6 hours. In a radical hysterectomy, the uterine arteries are transected at their inlets into the anterior division of the internal iliac arteries, and ureteric dissection is fairly easy. In the present cases, all uterine vessels were preserved, and extremely meticulous dissection is unavoidable in this surgically inaccessible area of the funnel-shaped pelvis. In all novel types of surgery, procedure duration tends to decrease as it becomes more standardized. This would most likely be the case for UTx live donor surgery in the future.

Naturally, as in all live organ donations, our surgery involved a person, with no direct health benefit from the donation, being exposed to a surgical risk. In live kidney

and liver donation, the risks of major surgical complications (grade III) are ~4% (29, 30). In the present study, one donor developed a ureterovaginal fistula which necessitated hysterectomy (grade IIIb complication). The late appearance of the fistula indicates that the cause was partial damage, possibly by diathermy, followed by a gradual weakening of ureteral wall. This particular donor surgery, spanning >12 hours, was extremely difficult, owing to the absence of natural dissection planes, and involved an older donor. This ureteric complication in the present study and the perioperative ureteric laceration, which occurred in the original case in year 2000 (13), indicates that extremely gentle surgery should be used in close proximity to the ureters.

Two of the nine recipients lost their grafts during this 6-month period reported here. The cause of graft loss was escalating uterine infection in one case and acute thrombosis in the other. The lengthy procurement procedure (12 hours) and poor synchronization of donor and recipient surgeries, with a resulting very long anesthesia time for the recipient, may have been other predisposing factors behind uterine infection. Moreover, the vaginal-vaginal anastomosis sutures were placed in close proximity to the cervix in that case, for anatomic reasons. This may have negatively affected the normal cervical barrier to ascending infections, especially in an immunosuppressed recipient.

The recipient with acute uterine artery thrombosis was heterozygous for protein C deficiency, which does not exclude a patient from any type of clinical transplantation surgery. However, this mutation is associated with 3–6-fold increased risk of venous thromboembolism (31). It is unclear whether the complication in this case of increased risk for thromboembolism might have been avoided if more intense or prolonged anticoagulants had been administered (31) or if the total surgical time of >16 hours that the uterus and its vasculature are exposed to could have been considerably shortened. Other factors, which may have predisposed to thrombosis of this case, are that this transplantation involved the oldest donor and that the uterine artery blood flow, after anastomosis, was the lowest of all cases. The total failure rate of 2/9 should be viewed in the light of this being a novel type of transplantation, with major potential for further optimization.

Our immunosuppression protocol was milder than in the two previous cases of human UTx (13, 14) as well as in those initially used for hand and face transplantations (32). Nowadays, the general understanding is that less immunosuppression is sufficient in any type of composite tissue transplantation (33). The fact that we observed only mild rejection episodes in four of the seven recipients indicates that it is unlikely that immunosuppression was suboptimal.

Effective monitoring of each specific organ to detect any rejection at an early stage, when it is reversible and has not caused organ damage, is a major challenge in organ transplantation. Because UTx is a novel type of transplantation, the ideal mode for monitoring the organ is not known. In the first human UTx case in 2000, repeated Doppler examinations and CD4/CD8 ratios in blood were used to detect rejection (13). One rejection episode was detected and effectively reversed, but the uterus had to be removed later. In the more recent case from 2011, Doppler and biopsies of the graft's vaginal portion were used and no rejection episodes were reported (14).

The mild rejection episodes in our patients were asymptomatic, with normal results on ultrasound and gynecologic examination. We based our rejection diagnosis on the assumption that a human uterine allograft would react similarly to that of a baboon, in which we observed the wide spectrum from mild to severe rejection (6). The fact that corticosteroids were effective in normalizing the histologic pattern in our human cases indicates that these were true rejection episodes.

The plan for this cohort is to start embryo transfer 12–18 months after UTx if the clinical course has been uneventful, with no rejection episodes for ≥4–6 months. The uterus will be removed after one or two successful pregnancies.

In summary, this study shows that a live-donor UTx procedure has a low risk despite extended surgery duration. The report of the first successful human UTx case, defined as a live birth from a transplanted human uterus, has yet to be published.

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Uterus transplantation trial: 1-year outcome

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Objective: To report the 12-month outcome of seven patients with viable uteri after uterus transplantation (UTx).
Design: Prospective observational study.
Setting: University hospital.
Patient(s): Seven patients with absolute uterine infertility and viable uteri for 12 months after live-donor UTx.
Intervention(s): Predetermined immunosuppression was with tacrolimus and mychophenolate mofetil (MMF) during 6 months, whereupon MMF should be withdrawn. Frequent ultrasound examinations were performed to assess uterine appearance and uterine artery blood flow. Cervical biopsies (for histological detection of rejection) were obtained at preset time points, with temporary adjustments of immunosuppression if there were signs of rejection. Menstruations were systematically recorded.
Main Outcome Measure(s): Menstruation, uterine artery blood flow, histology of cervical biopsies, and blood levels of tacrolimus.
Result(s): All patients showed regular menses after 1–2 months. Uterine artery blood flow was unchanged, with a median pulsatility index of 1.9 (range, 0.5–5.4). Blood levels of tacrolimus were approximately 10, 9, and 8 (μg/L) during months 2, 9, and 12, respectively. Four recipients showed mild inflammation in biopsies after MMF withdrawal and were treated with corticosteroids and azathioprine during the remainder of the 12 months. Subclinical rejection episodes were detected on ectocervical biopsies in five recipients. Histology showed apoptotic bodies and occasional spongiosis in the squamous epithelium. Moderate infiltration of lymphocytes and neutrophils was seen in the epithelial/stromal interface. All rejection episodes were successfully treated for 2 weeks with corticosteroids or dose increments of tacrolimus.
Conclusion(s): We demonstrate long-term uterine viability after UTx, with continued menstruation and unaltered uterine artery blood flow. Subclinical rejection episodes were effectively reversed by temporary increase of immunosuppression.
Clinical Trial Registration Number: NCT01844362. (Fertil Steril® 2015;103:199–204. ©2015 by American Society for Reproductive Medicine.)
Key Words: Infertility, MRKH, rejection, transplantation, uterus

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Absolute uterine factor infertility (AUFI) is considered to be the only major cause of female infertility that remains untreatable. Uterus transplantation (UTx) has been proposed as one possible AUFI treatment. Recently, we initiated the first clinical UTx trial after more than a decade of animal-based research (1, 2). Nine AUFI women were transplanted with uteri from live donors, with the majority of donors being mothers of the recipients. The surgical technique involved isolation of the donor uterus, with substantial vascular pedicles to include parts of the internal iliacs bilaterally, and then transplantation into the recipient by vascular anastomoses to the external iliac vessels. The results of the surgery and postoperative period have been reported in detail (3). The main findings of our previous study included data on unexpectedly long (10–13 hours) surgery times for uterine retrieval with one surgical complication (ureteric-vaginal fistula) in a donor. Furthermore, two of the nine uterine grafts had to be removed during the initial months, with the causes being uterine vessel thrombosis and severe intrauterine infection.

It should be noted that experimental human UTx had been performed

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2 times before our UTx trial, but in both cases in the absence of research preparations by the teams. The first was a live-donor case performed in Saudi Arabia in 2000, with progressive uterine necrosis occurring during the initial months (4). The other experimental case of UTx was performed in Turkey 2011 and involved a uterus from a deceased donor (5). The patient underwent embryo transfer 18 months post UTx, and two early clinical pregnancies, which miscarried, were reported (6).

In the present paper we report on the clinical course during the first 12 months among the seven women of our UTx trial who have kept their transplanted uterus during this first post-transplantation year.

MATERIALS AND METHODS

Patients

The study was approved by the Human Ethics Committee of the University of Gothenburg and was registered in Clinical Trials.gov (NCT01844362). Written informed consent was obtained from all donors, recipients, and their partners. Nine patients, evaluated extensively by a multidisciplinary team, underwent UTx as reported by us in detail in our previous publication (3). Two uterine grafts were removed during the initial 6-month period. One was due to persistent intrauterine infection (recipient 2), and the other was because of bilateral uterine vessel thrombosis (recipient 9).

The characteristics of the patients, their donors, and the surgeries are given in detail in our initial publication (3). In the present report and in all future publications, the original numbering of the patients will be kept to enable the reader to follow the medical, psychological, and quality-of-life outcome of each individual patient. Thus, in the present publication, recipients 2 and 9 are excluded, since their uterine grafts were removed during the initial 6-month period. The remaining seven women who kept their transplanted uterus throughout the first postoperative year had a median age of 28 (range, 27–35) years, had been in a steady relationship with their partners for $\geq$3 years, and had before the transplantation undergone IVF to exclude couples with fertilization failure and also to cryopreserve embryos for transfer, when >12 months had passed after transplantation. Six of the remaining recipients (numbers 3–8), had AUFI because of congenital uterine agenesis, and one (recipient 1) had undergone hysterectomy due to cervical cancer. The transplanted uteri were from the mother in four cases (recipients 1, 4, 6, 7), and in the other cases from a sister (recipient 8), mother's sister (recipient 3), and family friend (recipient 5). The human leukocyte antigen mismatch in the seven cases varied between 1/0 (recipient 7) and 3/2 (recipient 9). Thrombosis prophylaxis was with acetylsalicylic acid (75 mg; Trombyl, Pfizer) once daily, and this continued during the entire 12-month period.

Immunosuppression

Immunosuppression followed a standardized protocol, which is used for kidney transplantation at our transplantation center, and the protocol has been described in detail elsewhere (3). In short, the recipients received induction with methylprednisolone (500 mg; Solu-Medrol, Pfizer) 10 minutes before uterine reperfusion, and depending on local availability, thymocyte antibodies were given as either thymoglobulin (IV, 2.5 mg/kg body weight; Genzyme) or ATG (IV, 5 mg/kg bw; ATG-Fresenius; Fresenius) just before UTx and at a second occasion 12 hours later. All recipients were continuously treated with tacrolimus (Prograf/Advagraf, Astellas) with the aim to lower the trough levels in two steps: 10–15 ng/mL during the first month, followed by 5–10 ng/mL from the second month and onward. Mycophenolate mofetil (MMF; Cellcept, Roche) was given (1 g) preoperatively by the oral route. Starting from postoperative day 1, MMF was administered twice daily, and the aim was to keep the MMF area under the curve (MMF-AUC) trough levels at 40–60 mg $\times$ hour/L. Owing to the potentially teratogenic effects of MMF, this treatment should be discontinued after 6 months, which is a time at least 6 months before the planned initial ET. The aim was to treat the patients with solely tacrolimus from month 7.

Follow-up

All recipients were monitored by regular clinical visits and laboratory examinations. The frequency of these was initially twice weekly, and after a month they were spaced to fortnightly visits during months 2–6 and then monthly visits. The clinical examinations comprised macroscopic inspection of the transplanted uterine cervix and vaginal rim as well as cervical cultures and biopsies. The biopsies were obtained at predetermined time points (1, 2, and 4 weeks and thereafter monthly), and the protocol also included biopsies at suspicion (discoloured cervix, abnormal vaginal discharge, fever, abdominal pain) of any pathological condition, such as infection or graft rejection. The histopathological examination of the cervix was graded according to our proposed rejection classification for the primate uterus (7). In the events of verified rejection on cervical biopsy, the immunosuppression was temporarily elevated. In the event the planned withdrawal of MMF after 6 months resulted in a cervical biopsy that was, per protocol, obtained 2 weeks after withdrawal of MMF or any later biopsy showed signs of accumulation of inflammatory cells, azathioprine (Imurel, Orion Pharma), and prednisolone (Prednisolon, Pfizer) were added as continuous immunosuppression. In the event of cervical intraepithelial neoplasia (CIN) on a cervical biopsy, a real-time polymerase chain reaction assay for typing of human papilloma virus (HPV), which included primers for HPV16, 18, 31, 33, 35, 39, 45, 52, 58, and 67 (8, 9), was used, after DNA had been extracted from cervical brushings by a MagNA Pure LC methodology after isolation with a total nucleic acid kit.

At each clinical visit, transvaginal and abdominal two-dimensional ultrasound (Flex Focus 400, BK Medical AB) were performed to assess the endometrial and myometrial thickness and echogenicity. Doppler ultrasound was used to evaluate the uterine artery blood flow on both sides. Waveform characteristics and measurement of blood flow velocity at peak systole (PSV) and peak diastole were obtained to calculate the resistance index (RI) and pulsatility index (PI).

Routine blood tests were performed to assess blood status and liver and kidney function and to detect infection. Tacrolimus levels were measured in whole blood by an automated chemiluminescent immunoassay developed for use on the ARCHITECT system (Abbott Scandinavia AB). The intra-assay coefficient of variation is <1.9%. Levels of MMF were measured by an enzyme multiplied immunoassay technique on a Horiba ABX Pentra 400 clinical chemistry analyser (Thermo Fisher Scientific Inc.).

All complications of the recipients that occurred were registered and classified according to the Clavien-Dindo classification of surgical complications (10). Herein complications are classified into four major grades, where grade I accounts for complications requiring pharmacological treatment with antiemetics, antipyretics, analgetics, diuretics, and electrolytes. Grade II includes treatments with drugs other than the ones allowed in grade I or blood transfusions and total parenteral nutrition. Grade III complications include surgical, endoscopic, or radiological intervention under (grade IIIb) or not (grade IIIa) general anesthesia.

Statistics

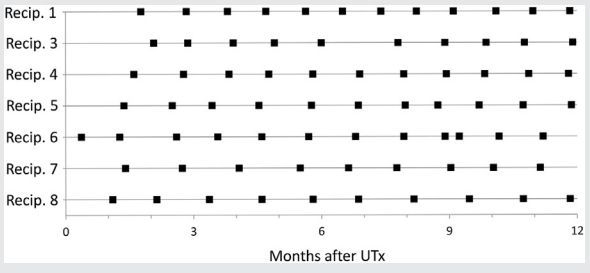
Quantitative variables are given as individual values and median and ranges.

RESULTS

Menstrual Pattern and Endometrial Thickness

Spontaneous menstrual bleedings occurred within 2 months in all seven recipients. Although some occasional irregularities appeared, regular menses with intervals between 27 and 32 days were seen in all patients (Fig. 1). The uterine appearances on two-dimensional ultrasound were unchanged during the study period. Typical cyclic changes of the endometrial thickness were seen, and the endometrium increased from 7.3 (3.2–10.4) mm (median [range]) during cycle days 5–8 to a maximum thickness of 14.4 (11.2–18.7) at cycle days 20–24. A trilaminar pattern was seen in all measurements done during the follicular phase, and a hyperechogenic pattern in those done during the luteal phase.

FIGURE 1



Menstrual bleeding (*black boxes*) in recipients 1 and 3–8 during the first year after UTx.

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Uterine Blood Flow

Doppler examination of the anastomosed uterine arteries showed normal uterine artery flow velocity waveforms in all patients and at all occasions. Calculated PI, RI, and PSV for each recipient and at multiple times showed only minor variations over time (Fig. 2), with median PI, RI, and PSV of 1.9 (0.5–5.4), 0.8 (0.3–1.4), and 25.4 cm/second (9.2–59.4), respectively.

Rejection Episodes

All rejection episodes were without any clinical symptoms, and the ectocervix had on all occasions a normal appearance on visual inspection. Rejection episodes were detected in five of the seven recipients (Fig. 3), and eight out of nine of these episodes were classified as Clavien-Dindo grade II complications. The episodes were categorized as mild rejections, and in one case as a borderline rejection (second rejection episode of recipient 5). Morphologic analysis of biopsies from ectocervix showed occasional spongiosis in the basal layers of the squamous epithelium and sporadic apoptotic bodies among the squamous cells. There was an increased amount of leukocytes, predominantly neutrophils and lymphocytes, in the epithelial/stromal interphase.

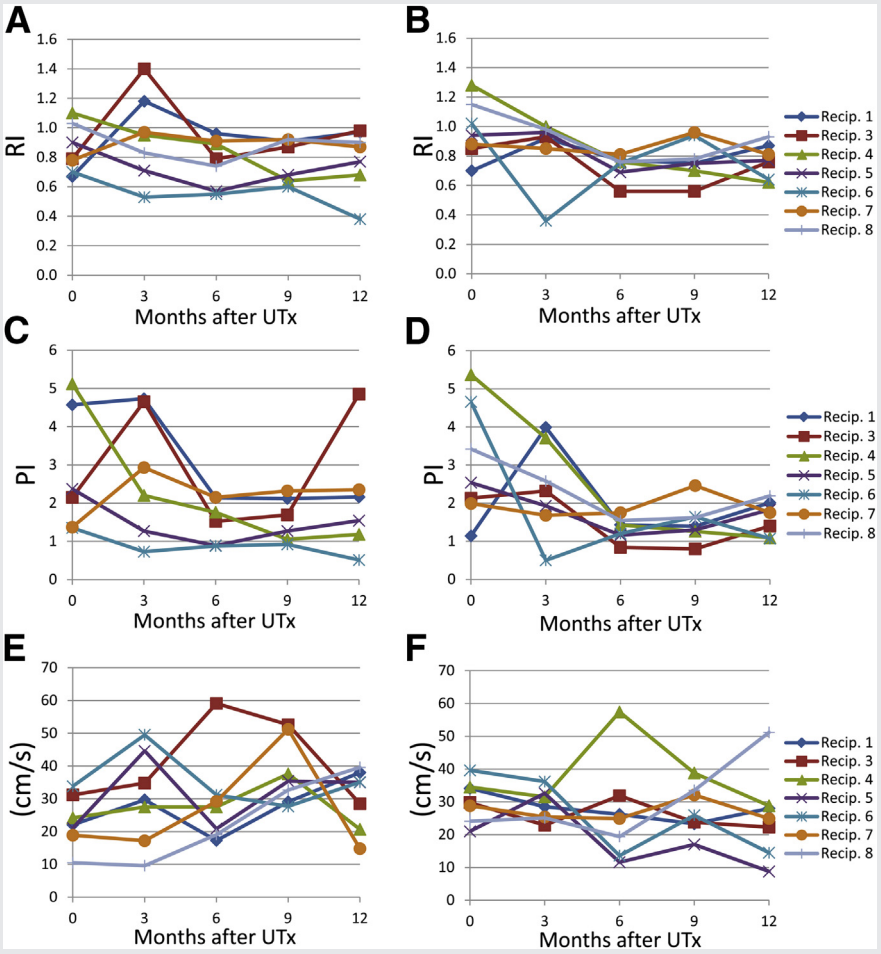
Two of the recipients showed three episodes of rejection each (recipients 5 and 8). With one exception, the rejection episodes were treated with corticosteroids (500 mg IV daily for 3 days; Solu-Medrol, Pfizer) and thereafter tapered oral corticosteroids for 7 days (Prednisolon, Pfizer). One episode of borderline rejection (recipient 5, episode two) was treated only with oral glucocorticoids. On all occasions of detected rejections, control cervical biopsies 2 weeks post–rejection treatment showed normal histopathology. All the rejection episodes were without any clinical symptoms, and the ectocervix had on all occasions a normal appearance on visual inspection.

In recipient 5, the per-protocol cervical biopsy obtained 8 months post-transplantation showed cervical intraepithelial neoplasia 2 (CIN 2), with the presence of HPV of subtype 31. A small loop excision, with inclusion of the biopsy site, was done under anesthesia 2 weeks later, and the event was thus classified as a Clavien-Dindo grade II complication. The cone biopsy showed normal epithelium and no signs of residual CIN. Subsequent biopsies have been normal.

Immunosuppression

The blood levels of tacrolimus are shown in Figure 3. The MMF-AUC in all seven patients was within the 40–60 mg × hour/L during the first 6 months, with a median of 47.0 (35.0–78.0) mg × hour/L. Occasional high and low values were shown during the initial month (data not shown). Withdrawal of MMF after 6 months was initially uneventful in four women (recipients 1, 3, 4, and 6), and monotherapy with tacrolimus was introduced. Recipient 3 presented with normal cervical biopsies during the initial 3 months after MMF withdrawal. However, mild inflammation was seen on the biopsy taken 9 months after UTx, and she was treated with the

FIGURE 2



(**A–C**) Doppler indices of *left* (**A**, **C**, **E**) and *right* (**B**, **D**, **F**) uterine arteries the first year after UTx. RI (**A**, **B**), PI (**C**, **D**), peak systolic velocity (cm/second; **E**, **F**).

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addition of azathioprine and prednisolone from that time point. In three recipients (recipients 5, 7, 9) the control biopsy 2 weeks after MMF withdrawal showed low infiltration of inflammatory cells, and thus azathioprine and prednisolone were introduced already at this stage. These recipients were then on triple immunosuppression during the remainder of the observation period.

DISCUSSION

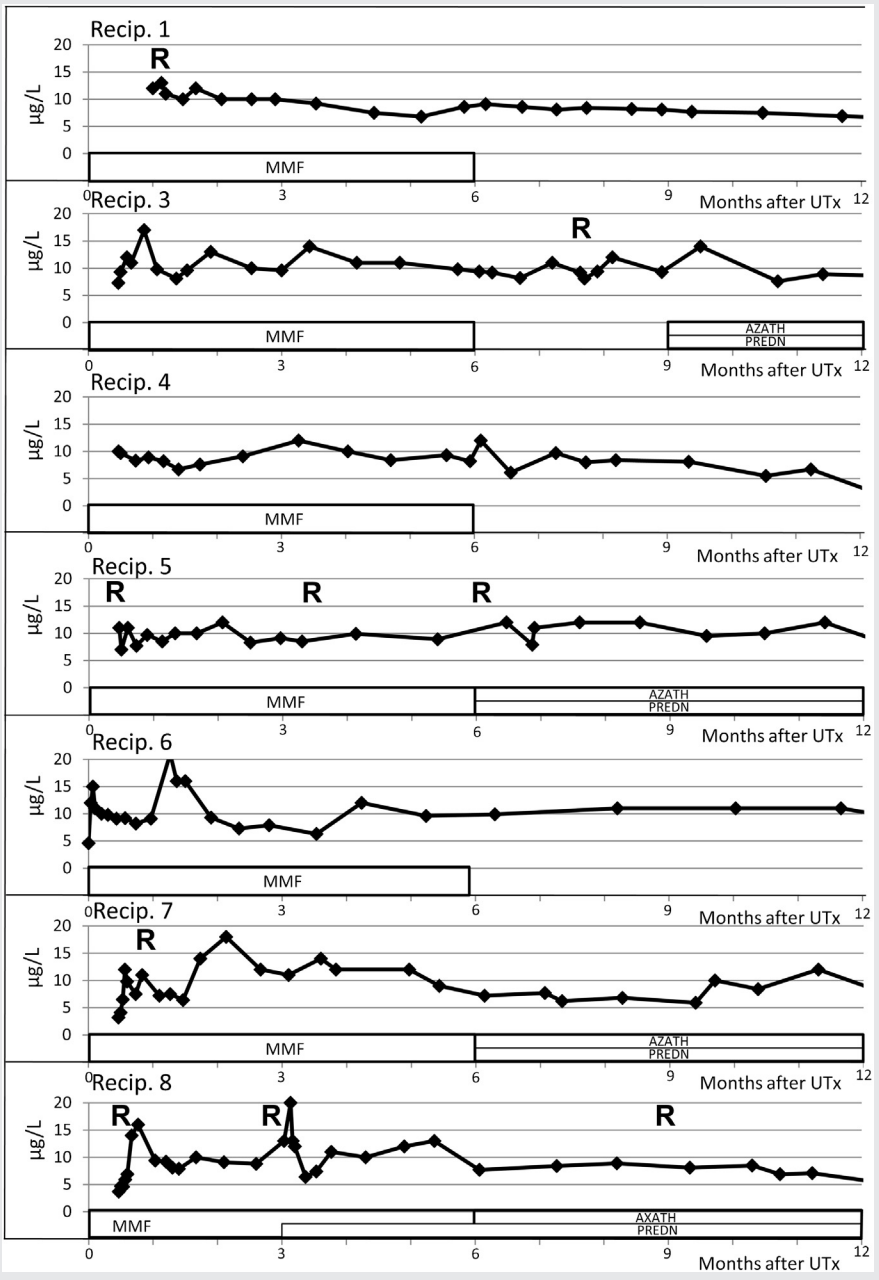
UTx, as a possible future treatment option for patients with AUFI, is presently at its clinical experimental stage. The seven patients of our prospective observational study and one previous experimental single case of UTx in 2011 make up the total worldwide cohort of eight UTx patients with viable uterine grafts today. Three additional UTx cases that have been performed ended in removal of the uterus. Two of these unsuccessful cases were recipients of our study, with removal of a uterus with thrombotic uterine vessels within 1 week post UTx in one case and hysterectomy after 4 months in the other case. That hysterectomy was because of persistent infection inside the uterine cavity that was resistant to IV antibiotic treatments and multiple attempts of surgical drainage.

It is naturally of great importance that the ongoing human UTx cases are closely followed to gain all possible information that may be beneficial for the design of future research studies in the field and of course in the long run for the large group of women worldwide who have AUFI. Our aim is to publish scientific reports at different intervals after UTx and to cover all aspects of the procedure.

The main findings of the present study were that all seven patients who had an uneventful postoperative recovery during the first few months also did well during a 1-year time frame. Importantly, menstrual patterns and uterine blood flow were normal. Rejection episodes could be detected by the aid of cervical biopsies and effectively reversed by increased immunosuppression during a short time.

Spontaneous menstruations occurred within 2 months in all seven patients, which was expected, considering their age and normal ovarian function. In the experimental live-donor UTx case in Saudi Arabia (4), estrogen and P were given

FIGURE 3



Blood levels of tacrolimus during the first postoperative year after UTx and schematic overview of additional immunosuppressive medication with MMF, corticosteroids (PREDN), and azathioprine (AZATH) in each recipient are shown. Rejection episodes are indicated by R.

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during the first 3 months and two withdrawal bleedings followed cessations of the hormone therapy. Also in the experimental deceased donor UTx case in Turkey (5), initial menstrual bleedings were induced by sequential hormone therapy, with the first menses appearing 20 days post-UTx. In light of the early appearance of spontaneous menstruations in our seven uneventful cases, it is incomprehensible why hormone therapy had to be used in the two single and highly experimental cases, which included recipients aged only 26 (4) and 22 (5) years, with presumably good ovarian reserves.

Rejection of any transplanted organ is commonly diagnosed by findings of decreased function of the organ and/or typical changes of tissue biopsies. Considering the uterus, menstruation is the only obvious functional parameter that can be easily monitored by the patient. In our study, the rejection episodes were not accompanied by any obvious menstrual irregularities or any other clinical signs.

Before our UTx trial we had some, but limited, knowledge from our animal research studies of the means to detect rejection of a uterine graft. In the allogeneic mouse model we described the uterine rejection patterns, both in relation to gross morphological changes and blood flow (11) as well as of what specific subtypes of leukocytes invade the myometrium at rejection (12). However, collection of myometrial biopsies would be a difficult and highly invasive procedure after human UTx. Consequently, we based our rejection diagnosis in the human UTx trial on histological examinations of cervical biopsies. The assumption that the morphology of the cervical biopsies at rejection would mimic the morphology of the uterine body was based on results of our study on allogeneic UTx in the baboon (7, 13). In these studies, the morphology of cervical biopsies correlated well with the clinical picture of rejection, in the wide spectrum from mild to severe rejection. Additionally, the morphological analysis of the biopsy was not influenced by the cyclic changes of ovarian hormones or menstrual bleedings, and all the rejection episodes were reversible with increased immunosuppression.

In the present study a total of nine rejection episodes, restricted to five patients, occurred during the first postoperative year. All episodes were successfully resolved by temporary therapy with glucocorticoids. It is noteworthy that all the rejection episodes of the UTx trial were subclinical and asymptomatic and only detected by the cervical biopsy.

To possibly detect rejection or other uterine pathologies we applied two-dimensional ultrasound to examine the uterine size and echogenicity, as well as Doppler for measurements of uterine blood flow. Ultrasound examination has previously been tested as a means for detection of rejection after kidney transplantation, but it was found that this imaging method has limited value in diagnosing renal rejection (14). In our study, PIs were also within normal ranges according to the moment of the menstrual cycle (15). This would reflect that uterine vascularization is not compromised in the transplanted uterus, at least in a nonpregnant state.

The immunosuppression protocol used in the UTx trial is a standard immunosuppression protocol used in the transplantation of solid organs such as the kidney and the liver. In the experimental deceased donor UTx case in Turkey (5), the doses of immunosuppression were considerably higher than in the present study, with trough levels of tacrolimus being around 3-fold higher than in the present study. Moreover, MMF and corticosteroids were administered at high doses during the entire first year after UTx, which is in contrast to the short treatment with corticosteroids and the 6-month MMF treatment that were used in the present study. Even if UTx is an ephemeral transplantation, it is of importance to minimize the quantity of immunosuppressive medications to the patient to avoid serious dose- and time-related side effects.

The levels of immunosuppression used in the UTx trial together with the low numbers of rejection episodes indicate that the protocol used is sufficient to effectively suppress the immune system to avoid damage to the grafted uterus.

Importantly, a fetus growing in a transplanted uterus will unconditionally be exposed to the immunosuppressive treatment. As of 2006, more than 14,000 children born from immunosuppressed organ transplanted women have been reported, and no increased incidence of congenital malformations has been detected (16).

The predetermined plan for the seven women with viable uteri in this study is to start ET 12–18 months after UTx given an uneventful clinical course, with no severe rejection episodes for ≥ 4 months. After completion of one or two successful pregnancies, the uterus will be removed.

In summary, the results of the present study demonstrate long-term uterine viability and function after live-donor UTx. Asymptomatic rejection episodes can be detected by cervical tissue biopsies and resolved by temporary addition of glucocorticoid treatment.

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Livebirth after uterus transplantation

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Summary

Background **Uterus transplantation is the first available treatment for absolute uterine infertility, which is caused by absence of the uterus or the presence of a non-functional uterus. Eleven human uterus transplantation attempts have been done worldwide but no livebirth has yet been reported.**

Methods **In 2013, a 35-year-old woman with congenital absence of the uterus (Rokitansky syndrome) underwent transplantation of the uterus in Sahlgrenska University Hospital, Gothenburg, Sweden. The uterus was donated from a living, 61-year-old, two-parous woman. In-vitro fertilisation treatment of the recipient and her partner had been done before transplantation, from which 11 embryos were cryopreserved.**

Findings **The recipient and the donor had essentially uneventful postoperative recoveries. The recipient's first menstruation occurred 43 days after transplantation and she continued to menstruate at regular intervals of between 26 and 36 days (median 32 days). 1 year after transplantation, the recipient underwent her first single embryo transfer, which resulted in pregnancy. She was then given triple immunosuppression (tacrolimus, azathioprine, and corticosteroids), which was continued throughout pregnancy. She had three episodes of mild rejection, one of which occurred during pregnancy. These episodes were all reversed by corticosteroid treatment. Fetal growth parameters and blood flows of the uterine arteries and umbilical cord were normal throughout pregnancy. The patient was admitted with pre-eclampsia at 31 full weeks and 5 days, and 16 h later a caesarean section was done because of abnormal cardiotocography. A male baby with a normal birthweight for gestational age (1775 g) and with APGAR scores 9, 9, 10 was born.**

Interpretation **We describe the first livebirth after uterus transplantation. This report is a proof-of-concept for uterus transplantation as a treatment for uterine factor infertility. Furthermore, the results show the feasibility of live uterus donation, even from a postmenopausal donor.**

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Introduction

Absolute uterine factor infertility is the only major type of female infertility that is still viewed as untreatable. The major causes are congenital absence of the uterus (Rokitansky syndrome, also called Mayer-Rokitansky-Küster-Hauser syndrome), previous hysterectomy, and severe intrauterine adhesions.[1–4] In the UK alone, more than 12 000 women of childbearing age are thought to have absolute uterine factor infertility.[5] The available motherhood options for women with this disorder are adoption (to acquire legal motherhood), or pregnancy in a gestational surrogate carrier to acquire genetic motherhood, followed by adoption to also acquire legal motherhood. However, surrogacy is not allowed in many countries because of ethical, legal, or religious reasons.

We have undertaken preclinical research into uterus transplantation for more than a decade, using a step-by-step logical developmental approach, in which we have used several animal species, ranging from rodents to non-human primates.[6,7] Recently, we initiated the first clinical trial of transplantation, involving nine women who received uteri from live donors. Two of the women had to undergo hysterectomy during the initial months, with the causes being uterine artery thrombosis and severe intrauterine infection.[8] The other seven women began menstruation during the first 2–3 months and the grafts remained viable, with regular menstruations during the first post-transplantation year. Occasional subclinical episodes of mild rejection were detected on cervical biopsies, which were effectively reversed by short courses of increased immunosuppression.

Except for our clinical trial of nine women, only two other human uterus transplantation efforts have been reported. The first case resulted in progressive uterine necrosis during the initial months, and a fully necrotic uterus was removed 3 months after transplantation.[9] The second case involved a uterus from a deceased donor being transplanted into a patient with Rokitansky syndrome.[10] The patient underwent embryo transfer 18 months after transplantation and two pregnancies that miscarried before gestational week 6 have been reported.[11] No further reports exist about this case.

In this report, we describe the clinical course of the first patient in our cohort who achieved a clinical pregnancy resulting in delivery of a baby.

Methods

Patient

In 2013, a 35-year-old patient underwent uterus transplantation at Sahlgrenska University Hospital (Gothenberg, Sweden) as part of our clinical trial of uterus transplantation in nine women with absolute

For **clinical trial** see http://clinicaltrials.gov/show/NCT01844362]

uterine factor infertility (ClinicalTrials.gov number NCT01844362). The study was approved by the regional ethics board of the University of Gothenburg. The donor, recipient, and her male partner had given their written informed consent. The 6-month outcomes of the study have been published.[8]

The recipient (blood group O+) was born with Rokitansky syndrome (Müllerian agenesis). She belongs to the atypical Rokitansky syndrome category[12] since she was also born with only one kidney and has vaginal and uterine aplasia. A functional neovagina had been created by self-dilatation. She was a non-smoker, was not on any medication, and had a body-mass index (BMI) of 21 kg/m². The recipient was, before transplantation and at several times during the post-transplantation period, informed that surgical removal could later be recommended for medical reasons, owing to rejection, surgical complications at caesarean section, or side-effects of immunosuppression. The ethics approval and consent forms state that the uterus should be removed after a maximum of two successful pregnancies.

The donor (blood group O+) is a close family friend of the recipient. At surgery, she was 61 years old. She is two-parous with two previous vaginal deliveries, at 26 years of age (birthweight 3000 g) and at 29 years of age (birthweight 3250 g). Both deliveries were spontaneous and at gestational week 41. She is a healthy non-smoker and her BMI at surgery was 20 kg/m². Menopause occurred around 7 years before transplantation. To ascertain menstrual functionality of the uterus before transplantation and to possibly increase uterine artery blood flow preoperatively, she was treated for 3 months with a sequential oral contraceptive pill, containing ethinylestradiol (30–40 µg daily) and levonorgestrel (50–125 µg daily). Bleedings occurred as expected.

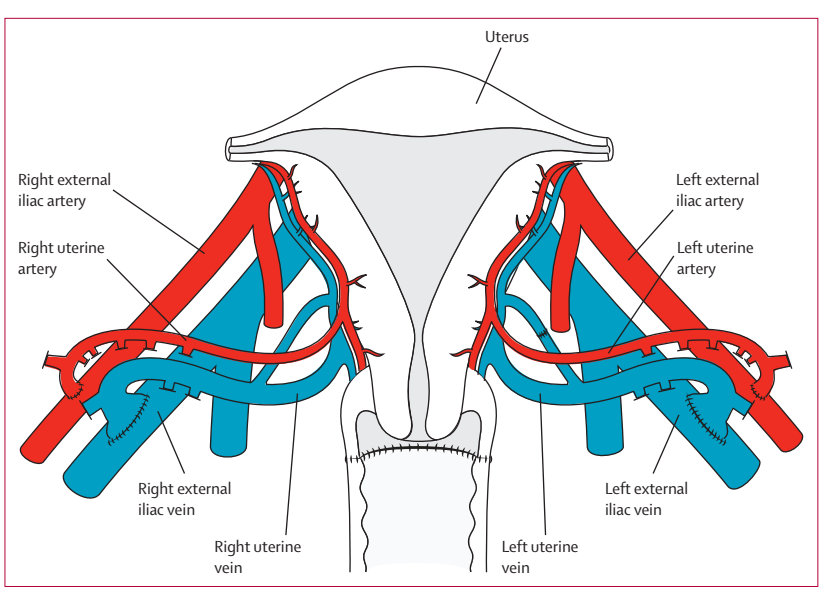

Figure 1: **Schematic drawing of the vessel connections of the transplanted uterus**

The HLA mismatch between donor and recipient was 3/2 and no HLA antibodies were present. Both donor and recipient were seropositive for cytomegalovirus and Epstein–Barr virus infections.

In-vitro fertilisation

In-vitro fertilisation was done during the period from 18 to 6 months before transplantation. The patient's Rokitansky syndrome, with no menstruation to aid in synchronisation of gonadotrophin stimulation and the cranially and laterally positioned ovaries, caused difficulties in initiation and monitoring of gonadotropin stimulation. Her blood concentration of anti-Müllerian hormone was 1·9 ng/mL. She underwent three full cycles of gonadotrophin stimulation. All cycles involved ovarian downregulation for 2–3 weeks by nasal administration three times daily with 300 µg of the gonadotropin-releasing hormone agonist buserelin (Suprecur; Hoechst, Frankfurt, Germany); this treatment began 7–9 days after a positive luteinizing hormone urine test, indicating the spontaneous gonadotrophin surge. We used ultrasound with abdominal probe and blood analysis of oestradiol values to assess follicle maturation. Human menopausal gonadotrophin (Menopur; Ferring, Copenhagen, Denmark) was used as the primary gonadotrophin in the first cycle (150 IU human menopausal gonadotrophin for 11 days) and recombinant follicle-stimulating hormone (Gonal-F; Merck Serono, Darmstadt, Germany) was added to the second and third cycles (225 IU human menopausal gonadotrophin plus 150 IU follicle-stimulating hormone, for 12 days in cycle 2 and for 14 days in cycle 3). Ovulation was triggered by injection of 250 µg recombinant human chorionic gonadotrophin (Ovitrelle; Merck Serono, Darmstadt, Germany). Oocyte pick-up was done transabdominally by abdominal ultrasound guidance. The oocytes were fertilised by intracytoplasmic sperm injection. Single embryo transfer was done around 12 months after transplantation during the natural menstrual cycle according to our local routine for frozen embryo transfer, with a soft embryo transfer catheter under abdominal ultrasound guidance.

Surgery

The surgical procedures of the donor and the recipient, and the anaesthesia, have previously been described in detail.[8] Uterus transplantation surgery entails isolation of the uterus with bilateral, long venous, and arterial vascular pedicles. The complexity of the surgery is mostly related to the extensive vascular dissection that includes the distal parts of the internal iliac veins and arteries. In this specific case, two large uterine veins on each side converged into one major uterine vein that drained into the internal iliac veins. On the patient's left side, one of these veins passed over the ureter and the other went under the ureter, and therefore one of these veins had to be transected to enable removal of the uterus with an intact ureter. After surgical isolation, the

uterus was flushed bilaterally through the arterial ends with cold histidine–tryptophan–ketoglutarate solution (Custodiol-HTK; NordMedica AS, Gentofte, Denmark). The vascular ends of the graft were trimmed and the left-sided vein that had been divided was anastomosed end-to-end by a continuous suture (8-0 polypropylene).

1 h before final graft retrieval from the donor, surgery to prepare the recipient for transplantation was initiated in an adjacent operating theatre. Through a midline incision, the external iliac vessels were dissected and prepared for anastomosis. The vaginal vault was separated from the bladder and rectum. Sutures, to be used for uterine fixation, were placed bilaterally through the round ligaments, sacrouterine ligaments, and the paravaginal connective tissues. The uterus was brought into the pelvis and end-to-side vascular anastomoses were done to connect the uterine veins to the external iliac veins (with 8-0 polypropylene sutures) and the anterior divisions of the internal iliac arteries to the external iliac arteries (with 7-0 polypropylene sutures) on both sides (figure 1). We then opened the blood flow to the uterus and ascertained that good pulses existed distal to the arterial anastomosis sites and that the uterine tissue changed from pale to reddish, which is a sign of peripheral tissue perfusion. Then, we fixed the uterus to the ligaments and sutured the extensive bladder peritoneum on the uterine graft on top of the recipient's bladder to provide extra structural support.

To establish that blood flow through the uterine arteries continued during the first post-transplantation days, we placed a 20-MHz Doppler probe with a silicon cuff (Cook-Schwartz Doppler probe; CookMedical, Bloomington, IN, USA) around the left uterine artery. The signal was transduced through a thin cable, which was exteriorised through the midline incision. The probe could then be easily pulled out after the 3-day observation period.

The surgeries of the donor and recipient proceeded uneventfully. The skin-to-skin durations of surgeries were 10 h 7 min for the donor and 4 h 55 min for the recipient. The anastomoses were created in the sequence of left venous, left arterial, right venous, and right arterial. After unclamping for reperfusion, we placed one extra suture over the arterial anastomosis site on the right side to prevent blood leakage. The estimated (Doppler) blood flow through the right uterine artery was then 40 mL per min. The uterus (figure 2A) was then attached to the orthotopic position (figure 2B). Perioperative blood loss was 0·6 L in the donor and 0·75 L in the recipient. The total ischaemic time of the uterine graft was 2 h 19 min (cold ischaemia: 1 h 6 min; warm ischaemia: 1 h 13 min). A retroperitoneal haematoma was diagnosed in the recipient on the second postoperative day and she was transfused with two units of leukocyte-reduced packed red blood cells. Both the donor and the recipient were discharged from the hospital after 6 days of postoperative care.

Immunosuppression and follow-up

The recipient received induction immunosuppression with intravenous anti-thymocyte globulin (Thymoglobulin; Genzyme, Cambridge, MA, USA) 2·5 mg/kg just before surgery and 12 h later. One dose of 500 mg methylprednisolone (Solu-Medrol; Pfizer, New York, NY, USA) was administered intravenously just before uterine reperfusion.

Maintenance immunosuppression was achieved with oral tacrolimus aiming at trough levels of 5–10 ng/mL (Prograf/Advagraf; Astellas Pharma, Chertsey, UK), and mycophenolate mofetil aiming at trough levels of 40–60 mg·h/L (Cellcept; Roche, Basel, Switzerland) was also administered orally during the first 10 months post-surgery. Azathioprine 2 mg/kg per day (Imurel; Orion Pharma, Sollentuna, Sweden) was then used instead of mycophenolate mofetil after 10 months, to avoid the potentially teratogenic effects of mycophenolate mofetil in the run-up to the embryo transfer attempts. Moreover, prednisolone (Prednisolon; Pfizer, New York, NY, USA) 5 mg daily was added from month 6 post-transplantation because of repeated rejection episodes (see Results section).

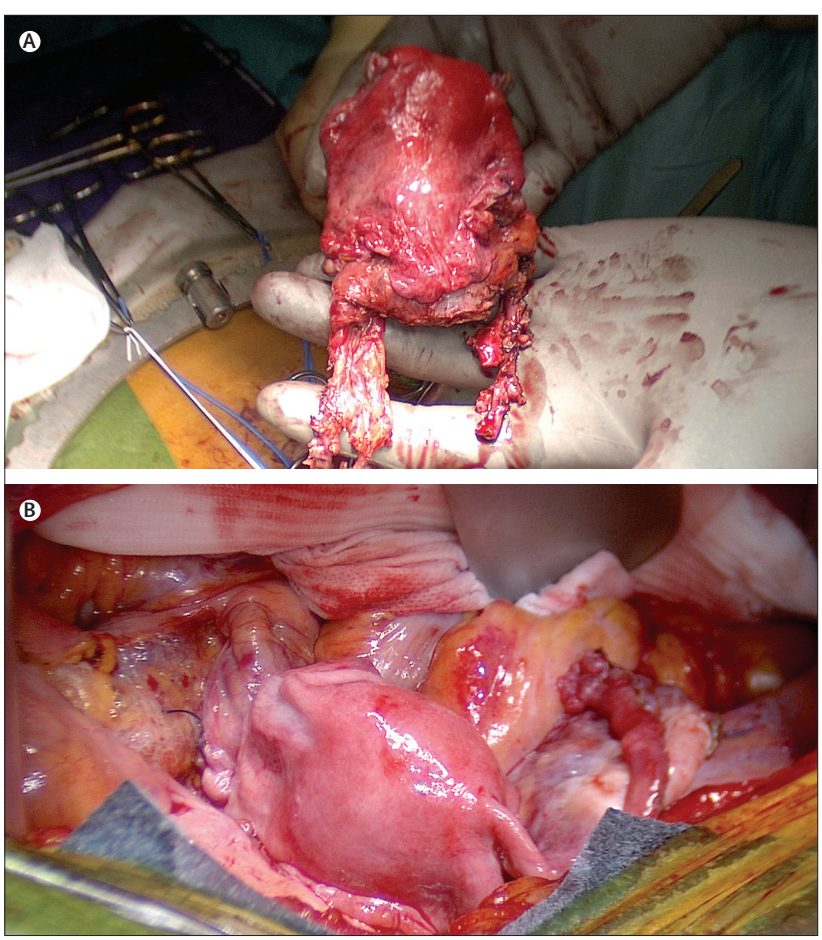

Figure 2: **Uterus transplantation procedure**
(A) The uterus with its long vascular pedicles is removed from the donor. (B) The uterine graft is revascularised and fixed in the pelvis of the recipient.

	Week 8	Week 12	Week 13	Week 15	Week 19	Week 25	Week 27	Week 29	Week 31
Weight (kg)	66	66	65	64	66	69	70	71	74
Cervical length (mm)*	..	..	44	43	45	48	50	49	31
Blood pressure (mm Hg)	100/75	100/75	110/60	110/60	110/70	120/75	110/70	120/80	170/105
Proteinuria (g/L)	0	0	0	0	0	0	0	0	1–3
Haemoglobin (g/L)	84	99	99	99	89	98	100	102	107

*Cervical length was not measured before week 13.

Table: **Maternal characteristics during pregnancy by completed week of gestation**

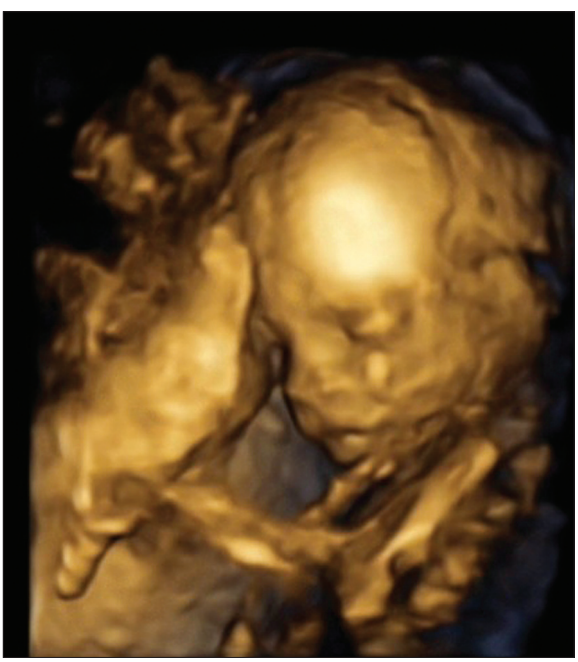

Figure 3: **3D image of the fetus' face at organ ultrasound screening in gestational week 18**

The recipient was followed up by frequent clinical visits and laboratory examinations, initially twice weekly during the first postoperative month and then every 2 weeks in months 2–6. Subsequently, she was seen monthly. The clinical examination involved a gynaecological examination with visual inspection of the transplanted uterine cervix, bacterial culture from the cervical canal, and occasional cervical biopsies. Ultrasound scans with transvaginal and abdominal probes were done to assess uterine size, and endometrial thickness and echogenicity. Uterine artery flow velocity waveforms on both sides were assessed by Doppler ultrasound, with the abdominal probe placed just above the inguinal ligament.

Biopsies of the uterine cervix were obtained at predetermined timepoints (at 1, 2, and 4 weeks, and monthly thereafter) and in the event of pathological signs (abnormal vaginal discharge, fever, discoloured cervix, or abdominal pain) that could be related to local infection or graft rejection. The histological examination of the biopsies used a uterine rejection grading system that was initially developed for the non-human primate uterus.[13] Any presence of cervical intraepithelial neoplasia was followed by tests for human papillomavirus.

Clinical follow-up also included monitoring of blood pressure and bodyweight, and laboratory monitoring of: serum creatinine and liver enzymes; blood haemoglobin, leukocytes, platelets, iron store, and glucose; urine albumin or creatinine; blood tacrolimus concentration (measured in whole blood by an automated chemiluminescent immunoassay; Abbott Diagnostics, Abbott Park, IL, USA) and concentrations of mycophenolate mofetil (measured by an enzyme multiplied immunoassay technique [Horiba ABX Pentra; Thermo Fisher Scientific, Waltham, MA, USA]).

During pregnancy, the patient was monitored according to Sahlgrenska University Hospital's routine programme for pregnant transplant patients, including frequent visits (every 2–3 weeks) to specialists in high-risk obstetrics and transplantation. Ultrasound data relating to the pulsatility index of the uterine arteries and umbilical artery and fetal growth were compared against data from the normal population.[14,15]

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

The first menstruation in the recipient occurred spontaneously 43 days post-transplantation and continued for 4 days. She then had regular menses with a median interval of 32 days and ranging between 26–36 days. The endometrium showed typical changes in median width (maximum 11·3 mm; range 7·9–15·3 mm). The blood flow velocity waveforms of the uterine arteries were similar on the left and right side and were within the low to normal range throughout the observation period. Median pulsatility indices were 1·27 (range 0·89–2·37) on the left side and 1·83 (1·30–2·54) on the right side.

Two mild rejection episodes (one after 9 days and the other at 6 months and 24 days) and one borderline

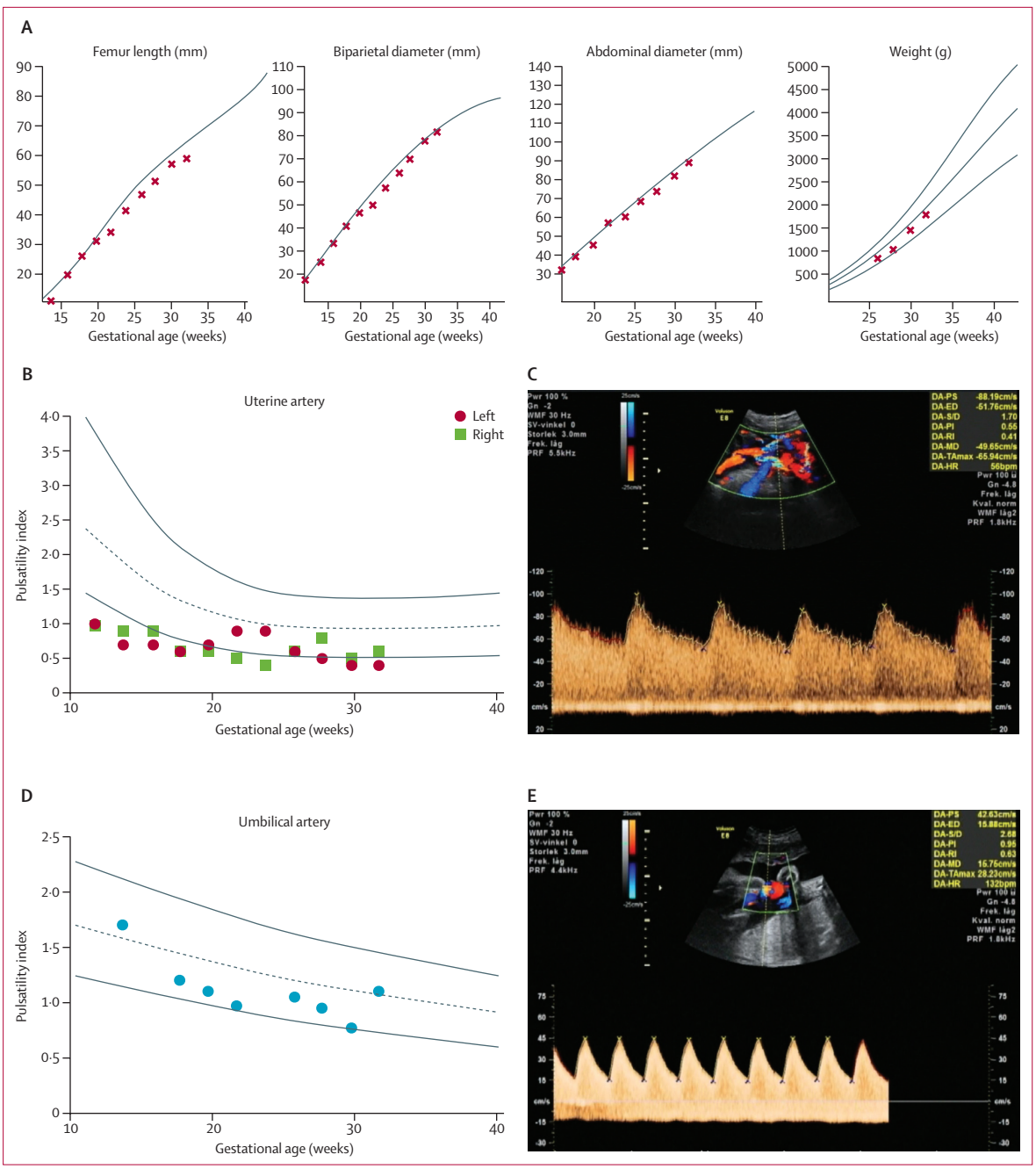

Figure 4: **Ultrasound estimates of fetal growth and flow velocity in maternal uterine arteries and fetal umbilical artery during pregnancy**
(A) The femur length, biparietal diameter, abdominal diameter, and estimated fetal weight were all within the normal range. (B) The uterine artery flow velocity waveform (pulsatility index) was low to normal on both sides; (C) shows a typical recording. (D) The pulsatility index in the umbilical artery was within the normal range during pregnancy; a recording is displayed in (E).

episode (at 2 months and 28 days) were diagnosed by cervical biopsies. These episodes occurred without any clinical symptoms. The rejection diagnosis was based on changed histology with a moderate increase in lymphocyte density in the stroma and the epithelium, with some spongiosis in the basal level of the epithelium. Occasional apoptotic cells were recorded in the basal epithelium together with reactive changes in the surface epithelium. The rejection episodes were successfully reversed by corticosteroid treatment (see later).

A biopsy 8 months and 12 days after transplantation showed moderate squamous epithelial dysplasia (p16 positive) and koilocytosis. Human papillomavirus typing

identified subtype 31. A follow-up mini-conisation 2 weeks later, with inclusion of the biopsy site, was normal and subsequent biopsies displayed no dysplasia or koilocytosis.

The median blood concentrations of tacrolimus were 9·5 ng/mL (range 3·0–12·0) during the first 3 months, 8·9 ng/mL (8·5–9·9) during months 4–6, 12 ng/mL (7·9–12·0) during months 7–9, and 10·0 (9·5–12·0) during months 10–12. The median blood tacrolimus concentration for the entire pre-pregnancy period was 9·8 ng/mL (range 3·0–12·0). The mycophenolate mofetil area under the curve was 51·3 mg·h/L (range 36·5–96·7) during the 10 months of treatment. The first mild rejection episode (on day 9) was treated with methylprednisolone 500 mg intravenously for 3 days, followed by oral prednisolone (starting dose 10 mg twice daily) for 4 weeks in a tapered protocol. The borderline episode (at 2 months and 28 days) was only treated with oral prednisolone in the 4 weeks-tapered protocol. The second mild rejection episode (at 6 months and 24 days) was initially treated in the same way as the previous mild rejection episode (see above) but the patient continued with oral prednisolone (5 mg daily) for the whole pre-pregnancy period. Since the recipient experienced two clear rejection episodes and because one was fairly close in time to the planned omission of mycophenolate mofetil, we decided that the patient would stay on mycophenolate mofetil for a total of 10 months and that azathioprine (2 mg/kg per day) would then be the replacement antiproliferative immunosuppression. Blood pressure and haemoglobin concentration during this initial post-transplantation year were stable at around 120/70 mm Hg and 100 g/L, respectively.

After in-vitro fertilisation, before transplantation, one cryopreserved embryo was obtained from one oocyte in cycle 1, four embryos from nine oocytes in cycle 2, and six embryos from eight oocytes in cycle 3. The embryo transfer was done 1 year after uterus transplantation and took place in the early luteal phase of the menstrual cycle. 3 days after a positive urinary luteinizing hormone test, three embryos were thawed, one of which was acceptable for transfer. The four-cell embryo had three surviving blastomeres. At embryo transfer, the endometrial thickness was 6 mm. The patient was on continuous treatment with 75 mg acetylsalicylic acid once daily since transplantation. She was treated with oral folic acid (250 µg twice daily) from 2 weeks before embryo transfer and vaginal progesterone (Lutinus; Ferring, Copenhagen, Denmark) 100 mg three-times daily for 9 weeks after embryo transfer.

A pregnancy test was positive 3 weeks after embryo transfer and 2 weeks later the intrauterine location and heartbeat of the fetus was detected by ultrasound. The pregnancy proceeded normally between 8 and 31 weeks of gestation—ie, the pregnant woman gained 8 kg in weight, cervical length was between 43 mm and 50 mm, haemoglobin was around 100 g/L (except for week 20 [see later]), blood pressure and blood glucose concentrations were in the normal range, and no proteinuria occurred (table). 3D ultrasound organ screening in gestational week 18 was normal (figure 3). Creatinine concentrations, in this patient with a single kidney, were somewhat raised during the pre-pregnancy period (median 94 µmol/L [range 80–111]) and were further elevated during pregnancy (106 µmol/L [86–147]), with creatinine concentrations constantly higher than 100 µmol/L from gestational week 27. Ultrasound examination then showed a slight hydronephrosis in the single right kidney. She was working full time until the day before delivery.

During pregnancy, growth in fetal femur length, biparietal diameter, abdominal diameter, and estimated weight were normal (figure 4A). The blood flow velocity waveform of the uterine arteries remained within the normal to low range during pregnancy (figure 4B, C). The pulsatility index of the umbilical artery was normal throughout pregnancy (figure 4D, E).

The patient was admitted to the obstetrics division at Sahlgrenska University Hospital (Gothenburg, Sweden) at 31 weeks and 5 days because of pre-eclampsia, with a blood pressure of 180/120 mm Hg, mild headache, proteinuria (urine albumin 18 mg/L), and lowered platelet count (96×10^9/L). She was given labetalol (200 mg three times daily) and nifedipine (10 mg twice daily), both administered orally, to reduce the raised blood pressure. Betamethasone (12 mg intravenously) was administered as respiratory distress syndrome prophylaxis. The patient had an increasing number of uterine contractions and cardiotocography showed occasional variable decelerations from around 10 h after admission. We applied continuous cardiotocography surveillance and because of ongoing repeated episodes of an abnormal cardiotocography

Figure 5: **The newborn baby just after birth**

pattern, a caesarean section was done 16 h after admission. The caesarean section was undertaken in spinal analgesia through a midline incision. Only mild adhesions existed. After careful dissection of the bladder peritoneum, with localisation of the major uterine vessels, a low-transverse uterine incision was made. The child was in breech position and was delivered 26 min after skin incision. The placenta (weight 375 g) was delivered immediately afterwards. Histological examination of the placenta showed a normal umbilical cord with tree vessels and no inflammation. The placental villi showed pre-eclampsia-like changes with villi of small calibre in relation to gestational length. Increased fibrin deposits and signs of fibrin thrombi were recorded in villous capillaries. Inflammation was not seen. After delivery, the uterus contracted well on intravenous oxytocin (10 IU). The uterine incision was sutured with a standard two-layered technique. A small myometrial biopsy was taken from the fundus uteri and the histology of this was normal.

The birthweight of the neonate was 1775 g, length was 40 cm, and head circumference 28·5 cm (figure 5). APGAR scores were 9, 9, 10 and the umbilical artery pH was 7·21. The first postnatal week was uneventful and the baby was in good condition, requiring only phototherapy and room air. The mother was in a good condition the day after delivery and her blood pressure was normalised spontaneously, with no further treatment. She was discharged from the hospital 3 days after caesarean section and is followed up in regular outpatient visits. The creatinine concentrations had decreased from 143 µmol/L on the day of delivery to 98 µmol/L 5 days later. The baby was discharged in good health from the neonatal unit 16 days after birth and the weight 21 days after delivery was 2040 g.

During pregnancy, the recipient continued with triple immunosuppression consisting of tacrolimus, azathioprine, and prednisolone. Tacrolimus concentrations tended to decrease during the first trimester and the tacrolimus dose was gradually increased to 40% of the pre-pregnancy dose (which was 10 mg daily) to achieve intended blood concentrations between 8 and 10 ng/mL. The median tacrolimus concentration during the entire pregnancy period was 7·3 ng/mL (range 4·6–13·0). During the first trimester, the patient presented with increasing leucopenia and anaemia, and the dose of azathioprine was consequently lowered from 2 mg/kg per day to 1·2 mg/kg per day during a period of 4 weeks. After 18 full weeks and 2 days of gestation, the protocol cervical biopsy showed mild rejection but the patient was asymptomatic. Inflammation was seen both in the subepithelial stroma and in the basal parts of the squamous cell epithelium (figure 6A). Apoptosis was also evident in the epithelium. No donor-specific HLA antibodies were detected at that time. She was treated with intravenous methylprednisolone for 3 days (250 mg on day 1, and 125 mg on days 2 and 3). The control biopsy after 20 full gestational weeks and 1 day (figure 6B) and the per-protocol biopsy after 30 full gestational weeks and 6 days were normal. The dose of azathioprine was then increased to its original dose (2 mg/kg per day) and the patient continued on this dose until delivery. The blood concentrations of tacrolimus during pregnancy were similar to those during the initial post-transplantation year (median 8·1 ng/mL [range 6·1–13·0]).

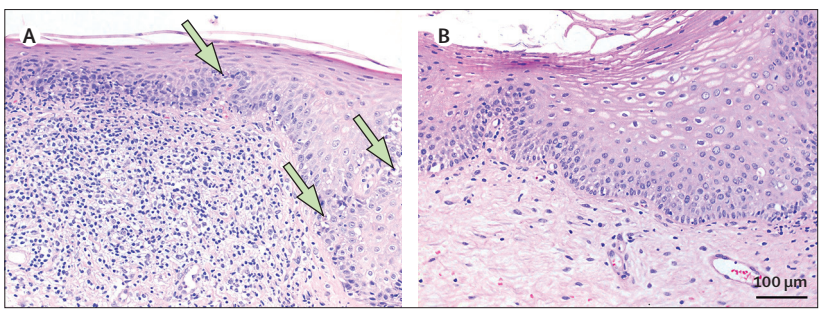

Figure 6: **Light micrographs from cervical biopsies showing rejection and normalisation 2 weeks after treatment**
(A) Biopsy showing mild rejection. A dense infiltrate of leukocytes, mainly lymphocytes, exists in the stroma and infiltrates into the basal layers of the epithelium, where occasional apoptotic cells can be seen (arrows). (B) 1 week later, after anti-rejection treatment, the leukocyte infiltration is almost completely reversed. The slides are stained with haematoxylin and eosin.

The haemoglobin concentration 3 days before embryo transfer was 101 g/L and the patient started treatment with oral ferrous sulphate 100 mg twice daily. In gestational week 8, haemoglobin fell to 84 g/L despite the ferrous sulphate treatment. One dose of 500 mg intravenous ferric carboxymaltose (Ferinject; Orifarm AB, Stockholm, Sweden) was administered and treatment with 60 µg weekly subcutaneous darbepoetinalfa (Aranesp; Amgen, Thousand Oaks, CA, USA) was initiated. Table 1 provides the blood haemoglobin concentrations throughout the pregnancy.

Discussion

Despite remarkable advances in infertility treatment, since the birth of the first in-vitro fertilisation baby in 1978[16] major forms of uterine factor infertility have remained untreatable. Our demonstration of a livebirth after uterus transplantation in a woman born with no uterus has eradicated the diagnosis of absolute uterine factor infertility.

This livebirth after human uterus transplantation comes after more than a decade of intensive animal research in this specialty by several groups worldwide. The ethical issues surrounding uterus transplantation are complex in their specific facets of non-maleficence, autonomy, beneficence, justice, and dignity. Modern uterus transplantation research began shortly after the first human hand transplantation in 1998,[17] which in many ways opened up the field of transplantation surgery to also include non-vital tissues or organs that after transplantation would have the chance to substantially increase an individual's quality of life. Thus, face and larynx transplantation have also now reached the stage as established clinical procedures.

Most types of organ and tissue transplantation that are done today use graft obtained from a deceased donor. The live donor concept is established for renal transplantation and for liver transplantation, but rates of live renal and liver donation vary greatly between countries. In the present study, the live donor was a close family friend of the recipient, by contrast with the other donors of our study cohort who were all family members. Our patient's first choice of donor was her mother, but blood group incompatibility prevented her from taking part in the study.

Uterus donation from live donors adds another key element into a risk–benefit analysis concerning uterus transplantation.[18] Uterine donation from a deceased donor would obviously substantially reduce the overall risks and complexity of the surgical procedure. In the uterus transplantation that was done in Turkey in 2011, the uterus was from a heart-beating, brain-dead, 22-year-old female, donor who had never been pregnant. Naturally, the young age of that uterus and its extensive vasculature would offer a benefit but this has to be balanced against the advantage of a uterine graft that has proved its functionality in terms of normal pregnancies. Moreover, the live donor concept allows for meticulous diagnostic workup of the uterine graft to exclude pathologies that could interfere with fertility potential, such as adenomyosis and endometrial polyps.

A specific concern with uterine donation is of course to exclude pathological disorders of the uterus that might be related to precancerous disorders. Both the donor and the recipient were human papillomavirus negative at our pretransplantation examinations and the presence of cervical dysplasia with human papillomavirus at 9 months after transplantation was unexpected. The reason for the temporary human papillomavirus positivity and secondary dysplasia is unknown.

A major reason to do in-vitro fertilisation before transplantation was that we needed to ascertain that fertility, in terms of fertilisation and initial embryo development, existed within the couple. Moreover, an in-vitro fertilisation procedure after transplantation might be more difficult than one before transplantation because of the abnormal uterine vascular pedicles and anastomosis sites that might increase the risk of bleeding at oocyte pick-up and because the immunosuppressed patient may have an increased risk of pelvic infection after the pick-up procedure. The first in-vitro fertilisation cycle of the patient only generated one embryo for cryopreservation and she was subsequently stimulated with very high gonadotrophin doses to obtain the normal oocyte yield. A previous report on surrogate in-vitro fertilisation outcome of patients with Rokitansky syndrome,[19] shows that women with the atypical Rokitansky form—as was the case with our patient—are poor responders and have a lower fertilisation rate than do women with the typical form of Rokitansky syndrome.

The patient became pregnant at her first transfer of a frozen-thawed embryo. The chance of that occurring in our setting and in her age group is around 16%.[20] Naturally, we did a single embryo transfer to avoid the risk of any multiple pregnancy, which would be an unnecessary additional obstetrical risk factor.

We diagnosed one episode of mild rejection during pregnancy. The histological rejection signs of the uterine cervix were reversible with a short course of increased corticosteroid treatment. The pregnancy itself induces a local immunosuppressed state and the myometrial and endometrial tissue of the uterine body might well have not shown the type of rejection-related inflammation that we diagnosed in the uterine cervix.

The pregnancy of our patient proceeded essentially normally for the first 31 weeks. The growth curves and the blood velocity waveforms of the umbilical cord were normal throughout pregnancy. In autologous uterus transplantation in sheep—which have pelvic vessels sizes similar to those in human beings—normal birthweights were also recorded.[21] Pulsatility indices of uterine arteries were within the normal to low range. A lower pulsatility index indicates decreased resistance of the blood vessels, which might well be caused by the denervated nature of the uterine graft and the absence of normal nerve-mediated vasocontrictive mechanisms. The normal to low pulsatility index of the uterine arteries would suggest a low, rather than high, risk of pre-eclampsia development. However, this specific situation of vascular supply from the external iliacs and with a transplanted uterus might ultimately reduce the predictive value of changes in uterine artery waveforms.

***Panel:* Research in context**

Systematic review

We searched PubMed for all publications with the search terms "uterus" AND "transplantation" AND "human", and we also ran a search for "uterine" AND "transplantation" AND "human". The searches included all papers, published in English language only, from 1956 up until Sept 30, 2014. The first search yielded 998 published papers and the second search provided 2228. All titles and abstracts, when available, were read to find out if they contained information about any human cases. Published data on 11 human uterus transplantation cases were found. The first human case (transplantation done in 2000) was reported in one research paper and the second case (in 2011) was reported in three research papers, covering surgery with 12-month follow-up, in-vitro fertilisation, and embryo transfer attempts with two early miscarriages, and one video presentation of surgical technique. The other nine cases were reported in our study detailing the surgery and 6-months outcome. Additionally, four reports existed on the surgical techniques of uterus retrieval from human deceased donors. The searches found nine articles about the ethics of uterus transplantation and 48 general reviews that at least partly covered the topic of human uterus transplantation.

Interpretation

Our present study is the first report of a livebirth after uterus transplantation and is thereby a proof of concept for this treatment of absolute uterine factor infertility. The efficiency of uterus transplantation as an infertility treatment is unclear and remains to be established. The livebirth rate of the complete cohort of the first clinical trial of uterus transplantation, which our patient belongs to, and results of future cohort studies will shed light on the efficiency of the procedure but also about what medical and psychological risks are involved.

The reason for the development of pre-eclampsia in this specific case is not known, but several plausible explanations exist. Immunosuppression might increase the risk of pre-eclampsia, and after kidney transplantation the pre-eclampsia rate is as high as 22%.[22] This situation is similar to the patient in our study, who also had one kidney and was on immunosuppression. The fact that a single kidney by itself could be an underlying factor of pre-eclampsia development is indicated by the fact that the rate of pre-eclampsia is about two-times higher in kidney donors than in their pregnancies before donation.[23] Other factors that might underlie the development of pre-eclampsia are the old age of the uterus, as indicated by the sevenfold increased rate of pre-eclampsia in women 50–60 years of age undergoing oocyte donation.[24] However, whether or not this increased rate is due to uterine factors is unclear since it can also be caused by age-related changes in other organs. Moreover, pre-eclampsia is more common in in-vitro fertilisation pregnancies[25] than after natural conception, and the total allogeneic situation, with a donated uterus or a donated oocyte, can increase the risk of pre-eclampsia.[26]

Our patient had periods of anaemia, leucopenia, and increased creatinine concentrations during pregnancy, which were probably immunosuppression-related side-effects that became apparent during pregnancy, with its added demands on several systemic functions. Thus, the potential side-effects of immunosuppression—such as nephrotoxicity (tacrolimus), bone marrow toxicity (azathioprine), diabetogenic effect (corticosteroids and tacrolimus)—should be taken in account in the planning of a pregnancy attempt after uterus transplantation, to decide the best possible time for embryo transfer in relation to immunosuppressive medication.

Uterus transplantation is the first ephemeral type of transplantation that has been introduced in which the graft is not intended for lifelong use. The uterus can be removed after one or two babies have been born, which would reduce the long-term side-effects caused by the immunosuppressive drugs. The patient of our present study had been informed that we can recommend surgical removal of the uterus before a second pregnancy attempt in the case of any major side-effects of immunosuppression. Such a decision should not be taken during the first few months after delivery, to allow for spontaneous reversion of side-effects that might have been aggravated by the pregnancy. Moreover, this delay would provide further observation time to ensure that the delivered baby is healthy and allow the uterus to return to its normal size, which would simplify any hysterectomy surgery. The autonomy of the patients should be respected and any future decision to surgically remove the uterus needs to be made in consensus with the recipient and her partner.

In conclusion, our demonstration of the first livebirth after uterus transplantation opens up the possibility to treat the many young women with uterine factor infertility worldwide.

Contributors

MB initiated the study, did surgery, followed up the patients, and wrote the report. LJ did surgery, followed up the patients, obtained data, and wrote the report. HB and HH followed up the pregnancy, obtained data, and wrote the report. NK did surgery, followed up the patients, and obtained data. JM did histological analyses, obtained data, and wrote the report. PD-K and MG did surgery, followed up the patients, and obtained data. AE did anaesthesia and obtained data. MM did in-vitro fertilisation and obtained data. JE followed up the patients, obtained data, and wrote the report. CD-G did surgery, obtained data, and wrote the report. AH did surgery and wrote the report. MO did surgery and wrote the report. LN did in-vitro fertilisation, obtained data, and wrote the report.

Declaration of interests

We declare no competing interests.

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