Uterus transplantation: an experimental study in the rat model

Musammad Shamima Nazmin Akhi

Department of Obstetrics and Gynecology
Institute of Clinical Sciences at Sahlgrenska Academy
University of Gothenburg,
Gothenburg, Sweden

UNIVERSITY OF GOTHENBURG

shamima.akhi@obgyn.gu.se

ISBN 978-91-628-8595-3
http://hdl.handle.net/2077/30561
Printed by Ale Tryckteam AB
Bohus, Sweden 2012

To my parents, brothers, and sister

whose support have sustained me throughout the life

and to Aurango

who made me laugh uncountable times.

Abstract

One of the last frontiers to conquer in infertility research is to find a treatment for uterine factor infertility, which affects around 2500 Swedish women. These women cannot become pregnant or carry a pregnancy due to absence of uterus or presence of non-functioning uterus. During recent years, several animal models have been used in research to develop uterus transplantation into a clinical treatment for uterine factor infertility. In the present study, the rat was used as a uterus transplantation model to look at various aspects of the procedure.

A first model for uterus transplantation in the rat, with vascular anastomosis, was developed. In this model, the native uterus was compared to a heterotopically placed grafted uterus within the same strain of inbred rats. There was good viability of the tissue and an untrained surgeon could master the procedure after around 20-30 surgeries.

In the second study, the uterus transplantation model was modified further to allow for spontaneous mating and test of pregnancy. Pregnancy was achieved after natural mating and the number of pups and growth trajectory of the pups in this model was similar to that of controls.

In tests of allogeneic uterus transplantation, effects of immunosuppression were evaluated. Transplanted rats received either no treatment or tacrolimus as monotherapy. One sham-surgery group and one sham-group treated with tacrolimus were included as controls. It was shown that rejection occurred in the non-tacrolimus treated transplanted group but that normal uterine morphology was seen in the tacrolimus treated transplanted group. Low numbers of T-cells were seen in most allografts treated with tacrolimus. Levels of the cytokines IL-1 and IP-10 were increased in the non-treated transplanted group and levels of the implantation marker galectin-1 were normalized after tacrolimus treatment.

Different sites of diagnosis of rejection were tested. In a fully allogeneic model, the histology of the graft was analysed at day 4 or 7. On day 4, morphological signs of early rejection were found both in the myometrium, endometrium, uterine cervix and in the blood vessels. Inflammation with primarily neutrophils and lymphocytes was seen. At day 7, the inflammation was greater with also focal hemorrhage. It can be concluded that early events of rejection in a uterus transplantation model is seen in all the examined compartments and the cervix may be an appropriate site for clinical diagnosis of early rejection.

The most important functional issue to test in uterus transplantation is whether uterine allografts can carry a pregnancy. Rats with allogeneic uterine transplants were treated

with tacrolimus. The pregnancy rate was similar in the transplanted and tacrolimus-treated group as in the control groups. These experiments ended during late gestation and no further follow-up of the pregnancy was performed.

In a follow-up paper of allogeneic transplantation, the pregnancies went to term. Birth weight was similar in the transplanted group that was treated with tacrolimus as in the control groups. The post-natal growth up to 100 days was also similar, but with somewhat larger weight for males born from the uterus transplanted group.

In summary, the thesis presents important background data for further development of uterus transplantation towards clinical introduction.

Keywords: infertility, microsurgery, pregnancy, rat, transplantation, uterus

List of Publications

I. **Uterus transplantation in the rat: model development, surgical learning and morphological evaluation of healing.**
Wranning CA, Akhi SN, Kurlberg G, Brännström M.
Acta Obstet Gynecol Scand. 2008;87:1239-47.

II. **Pregnancy after syngeneic uterus transplantation and spontaneous mating in the rat.**
Wranning CA, Akhi SN, Díaz-García C, Brännström M.
Hum Reprod. 2011;26:553-8.

III. **Uterine rejection after allogeneic uterus transplantation in the rat is effectively suppressed by tacrolimus.**
Akhi SN, Díaz-García C, El-Akouri RR, Wranning CA, Mölne J, Brännström M.
Fertil Steril 2013; in press.

IV. **Monitoring rejection after uterus transplantation: morphological assessment of different sites of a uterine allograft in a rat model.**
Akhi SN, Díaz-García C, El-Akouri RR, Brännström M, Mölne J.
In manuscript.

V. **First report on fertility after allogeneic uterus transplantation.**
Díaz-García C, Akhi SN, Wallin A, Pellicer A, Brännström M.
Acta Obstet Gynecol Scand. 2010;89:1491-4.

VI. **Live offspring after allogeneic uterus transplantation in the rat.**
Akhi SN, Díaz-García C, Brännström M.
In manuscript.

Table of Contents

Introduction

During the last 25 years, transplantation surgery and reproductive medicine have been particularly inventive clinical areas. In transplantation surgery, introductions of transplantation of organs/tissues, that have the potential to greatly enhance the quality-of-life of patients rather than being types of organs that are necessary for a continued life, have taken place. Examples of these novel types of procedures are transplantations of the hand, larynx, lower limb and the face (1). In reproductive medicine, several new techniques in the area of assisted reproduction techniques have followed the introduction of in vitro fertilization (IVF). The first IVF baby was born almost 35 years ago and the research behind this revolutionary infertility treatment was acknowledged by awarding the Nobel Prize in physiology and medicine in 2011 to Bob Edwards. The groups of infertile women that today, in spite of these developments in reproductive medicine, are untreatable are those that are irreversibly infertile due to uterine cause. The theme of this thesis is research on uterus transplantation (UTx), which is a type of transplantation procedure which may provide a chance for these women to carry their own child throughout pregnancy, but only if research paves the way for its clinical introduction.

General infertility

The inability to conceive a child is an important negative quality-of-life aspect for most women (2) and infertility is also categorized by WHO as a disease. A couple has met the criteria of being infertile if the woman has not become pregnant after 2 years of regular intercourse with no use of contraception. In many societies, including most parts of the western world, the term infertility usually refers to a 12 months involuntarily childlessness after contraceptive-free intercourse

Infertility is generally divided into primary infertility, when there has never been a child born within a couple, and secondary infertility, when there is a failure to conceive following a previous pregnancy within the couple. It is problematical to exactly estimate the prevalence of infertility, but involuntarily childlessness is usually estimated to be present among around 15% of all couples (3). It is stated that around 30% of the infertility causes are due to male factor and 30% to female factor. Around 10-15% of causes are due to combined factors in both male and female and the rest are still categorised as unexplained infertility.

Male factor infertility (4) is due to either poor sperm quality or low sperm count, which is usually the case after an obstruction of male reproductive duct.

Female infertility can be due to dysfunction on either the hypothalamic/pituitary level, the ovarian level or the uterine/cervical/vaginal level. Examples of rather common

hypothalamic/pituitary and ovarian disorders causing female infertility are hyperprolactinemia and polycystic ovarian syndrome. They usually cause infertility since they give rise to ovulatory failure. Beside these ovulatory disorders, the most common causes of female factor infertility is tubal factor infertility, which occurs in around 26% of all infertile women, endometriosis in about 4% of all infertile women, mucus abnormalities in around 4% of all infertile women and genital tract disorders in around 4% (5). Most of these causes of infertility as mentioned above are today treatable.

Uterine factor infertility

The type of infertility, which today is largely non treatable is uterine factor infertility. This group includes those women that have no uterus at all or those with a uterus but which is not functioning properly in terms of pregnancy potential. Infertility could also be defined as result of either structural or functional disorder of the uterus.

Uterine factor infertility is relatively uncommon in comparison with other groups of infertility, but since no treatment has been available the accumulated numbers of these patients of fertile age is relatively high. Thus, in the United Kingdom, with a population of more than 60 million people, there exist an estimated 14000 uterine factor infertile patients (6), and this figure would correspond to around 2300 in Sweden and around 160 000 uterine factor infertile women in Europe.

The largest group of women with uterine factor infertility is most likely those that have been hysterectomized, which during the fertile period may be performed because of malignancy, leiomyoma or as a life-saving procedure at intractable obstetric bleeding, secondary to uterus atony or malplacentation. In an IVF-surrogate program in USA, around half of the enrolled women with uterine infertility had been hysterectomized (7), indicating the considerable size of these hysterectomized women among the uterine factor infertile patients.

One cause of hysterectomy during fertile age is cervical cancer, which worldwide is the most common gynaecological malignancy (8), but lower incidences are seen in countries with cervical cytology screening programs. Around 30-40% of cervical cancer patients are of fertile age at diagnosis (8, 9). Tumors of low stage can be treated by uterine-sparing surgery (conization, trachelectomy) but radical hysterectomy is the recommended treatment for larger stage Ib and stage IIa tumors. The ovaries can be spared in squamous cell carcinoma of the cervix because the risk of ovarian metastasis is very low.

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 at delivery is around 5 in 10 000 deliveries (10). It is likely that this rate will increase in the future due to the escalating number of women having caesarean section.

Another cause of hysterectomy at fertile age is large or inoperable leiomyoma. However, as discussed further below most leiomyoma are small and do not cause any symptoms and if they are symptomatic, only the leiomyoma may be removed without removing the entire uterus. The prevalence of uterine leiomyoma increases with age (11). There exist reports of prevalence figures among reproductive-aged women of selected populations, as high as 20-40% (12). However, a more true prevalence may be that of around 5.5%, which was reported in a random sample of more than 300 Swedish women between 25 and 40 years of age (13). In that study, a higher prevalence (8%) was seen in the subgroup of older women between 33 and 40 years of age. In the United States, a comparable prevalence was seen in Caucasian women but with a 2-fold higher prevalence in Afro-American woman (14). In the United States, approximately 1% of all women between 30 and 34 years and around 2.5 % of those between 35 and 39 years have been hysterectomized due to leiomyoma (15). This is naturally one group of leiomyoma-related uterine factor infertility. However, more often leiomyoma will lead to infertility in a woman that still has her uterus (16, 17). It is difficult to exactly understand the causation between presence of leiomyoma and pregnancy outcome. It may be that there is a structural cause, with the leiomyoma preventing implantation and pregnancy progression by its physical size or placement within the uterus but also biochemical local factors have been suggested to contribute to leiomyoma-related infertility (18). In a thorough review of a large IVF population it was shown that it may be that larger sized myoma (> 4 cm) that may decrease fertility (19). Moreover, smaller subendometrial myoma may be a factor behind uterine factor infertility.

Some of this leiomyoma-related infertility can be surgically treated by myomectomy (20). The patients that remain infertile despite myomectomy and the large numbers that have undergone hysterectomy because of large symptomatic leiomyoma belong to the group of leiomyoma-related uterine infertile patients that could be treated by UTx.

Another cause of uterine factor infertility is intrauterine adhesions, where the endometrial cavity is totally or partly obliterated due to that opposing sides of the cavity is connected by adhesions. The prevalence of intrauterine adhesions is around 1.5% among fertile-aged females (21). Intrauterine infection is the most common cause of severe intrauterine adhesions (22). Other causes are surgical curettage at legal abortion or post partum (23). In general, intrauterine adhesions result in infertility in around 50% of women and if

pregnancy takes place the rate of spontaneous abortion is around 40% (24). Intrauterine adhesions can to some extent be treated by hysteroscopic adhesiolysis, with postsurgical fertility in around 90%, 70% and 30% of preoperative mild, moderate and severe intrauterine adhesions, respectively (25).

One larger group of uterine factor infertile patients are those with Müllerian duct anomalies. The Müllerian ducts are of mesodermal origin and are primordial roots of the internal female reproductive organs. They differentiate to form the Fallopian tubes, uterus, uterine cervix and the upper 1/3 of the vagina during fetal life.

In a large study by Grimbizis and colleagues, data from multiple studies on uterine anomalies including more than 3000 patients calculated the incidence of uterine malformation in the general population to be around 4.3% (26). The incidence of Müllerian duct anomalies is probably somewhat higher in women with infertility and in those having recurrent spontaneous abortion incidences of up to 15% have been reported (27, 28). In a study of more than 2000 girls undergoing ultrasound examination the presence of uterine anomalies was around 4/1000 women (29).

The American Fertility Society has classified the Müllerian anomalies into 7 specific groups, where group 1-6 are due to genetic or epigenetic causes and group 7 is related to fetal exposure to diethylstilbestrol. Clinical diagnosis of the anomalies is often carried out with a combination of clinical examination, ultrasound, laparoscopy and magnetic resonance imaging (30).

The most prevalent type of structural congenital uterine anomaly among infertile women is the septate uterus (31), which is the result of incomplete resorption of the central parts of the Müllerian ducts after fusion. The septate uterus makes up around 1/3 of all uterine malformations (26). Spontaneous abortion is seen in about 80% of pregnancies in untreated septate uteri (32). Hysteroscopic resection is however an effective treatment that substantially decreases the rate of spontaneous abortion (32). Even so, a small proportion of patients with surgically treated uterine septate will still remain infertile (33).

The second most common type of Müllerian duct anomaly is the bicornuate uterus, where disturbed fusion of the Müllerian ducts gives rise to bilaterally fully developed uterine horns with a single cervix and vagina. The malformation represents around 1/4 of all uterine malformations (26). The rate of spontaneous abortion among women with bicornuate uteri is around 35% (34). Surgery may normalize the increased rate of spontaneous abortions (35), but a large number of women with bicornuate uteri will not be able to carry a pregnancy to the second or third trimester.

The less common unicornuate and uterus didelphys comprise around 20% of uterine malformations (26, 36). Disturbed development of one of the Müllerian ducts will result in the unicornuate uterus, with or without a contralateral rudimentary uterine horn. Such a rudimentary horn could be either communicating or non-communicating. It should be noted that there often is ipsilateral renal agenesis on the side of the rudimentary horn (28). In presence of a unicornuate uterus, there is higher obstetric risk and this anomaly is associated with increased risk of preterm labour (43%), spontaneous abortion (34%) and ectopic pregnancy (4%)) (37, 38). It is important to surgically remove a uterine rudimentary horn if that contains functional endometrium and this could be done by a laparoscopic hemihysterectomy (39).

A total failure of fusion of the Müllerian ducts results in uterus didelphys, i.e. two separate uterine horns without a common cavity. The duplication of the vagina and the cervix may be partial or complete. The usual form is that of two separated uteri but with the endocervical channels fused at the distal end. The potential to establish a pregnancy in these two types of malformed uteri is decreased and in case of pregnancy around 30% will end in miscarriage and the total live birth rate is only around 50% (26). Surgery does not seem to improve the pregnancy potential of the unicornuate/dideplhys uterus (40). However, there exist reports of simultaneous pregnancies in each didelphic horn, with long intervals between deliveries (41) that would indicate origin in different ovulatory cycles.

The most extensive type of Müllerian duct anomaly is uterine agenesis, which represents around 3% of all these congenital uterine malformations (26) and it is seen in around one in every 4500 females (42). Typically these women have a rudimentary solid bipartite uterus in combination with absence of the vagina above the hyminal ring. The syndrome is generally named the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome or only the Rokitansky syndrome. Three subtypes of the Rokitansky syndrome exist. The typical subtype, which goes without extrauterine malformations, is present in around 50% and the atypical subtype, with associated malformations in the renal system, is present in around 20%. In the remaining 30% of the patients a severe form of Rokitansky syndrome exists with malformations also in the skeleton of the upper back and neck (43). The outcome of gestational surrogate pregnancies in Rokitansky patients as the genetic mothers (44) does not demonstrate any increased malformation risk in the offspring.

Rejection

Prevention of rejection, by using effective immunosuppression is a main focus of this thesis which is about UTx. Transplantation of any organ or tissue allograft stimulates an immune response in the host, directed against the donor tissues. The extent of this immune response to a graft may depend on the degree of genetic disparity between the

donor and the recipient. Clinically and depending on the onset of tissue destruction, rejection can be categorized into 3 groups: hyper acute, acute and chronic rejection.

Hyperacute rejection

Hyper acute rejection can be defined as very early graft destruction that appears within minutes to some hours after connecting the graft to the blood vessels. This type of rejection occurs in individuals with preformed antibodies against major allograft antigen**.** These preformed antibodies may be specific for ABO blood group antigen or they may be for allogeneic MHC molecules that have been developed during a prior exposure of the recipient to allogeneic cells due to for example blood transfusion, pregnancy or previous organ transplantation (45). In a UTx situation, the most common origin of this is likely to be previous transfusion, since the procedure would be restricted to previously infertile women and previous organ transplantation, this should exclude the patient from another major surgical trauma such as UTx. The preformed antibodies bind to the antigens in the vascular endothelium of the graft and activate the complement and clotting systems, thereby destroying the endothelial lining of the graft and this may lead to immediate thrombus formation (45).

The morphologic spectrum resulting from hyperacute rejection has been described for renal and cardiac transplants in experimental rats and primates (46, 47) as an edematous, mottled, and cyanotic graft within minutes to hours of vascular anastomosis. Hyper acute rejection is not common in the clinical settings, because every individual is tested for blood type and for antibodies against the cell of potential donor. This test is known as cross-match.

Acute rejection

Acute rejection usually occurs within weeks to month of transplantation and is the major immunological risk for developing allograft dysfunction. This process is considered primarily a T-cell mediated process (cellular mechanisms) although humoral (antibody mediated) mechanisms also contribute. In this T cell-dependent pathway to rejection, donor alloantigens are processed by specialized cell known as antigen-presenting cells (APCs), which by activation of alloreactive recipient´s T-cell leads to damage of the graft.

Patient with acute cellular rejection may be without clinical signs or symptoms but often present with sudden deterioration in allograft function. Most of the immunosuppressive drugs that are currently used are designed to prevent acute allograft rejection by depleting the recipient´s T-cell. Evidence of acute rejection occurs in the majority after organ transplantation but it can be reversed with adjustment of immunosuppressive therapy.
The immunological mechanisms involved in acute rejection are fairly well characterized.

This type of allograft rejection involves three consecutive steps. At first there is recognition of alloantigen by the recipient´s T-cells and this is followed by activation of T cells. Finally, these activated T cells attack and destruct the allograft. Antigens, antigen-presenting cells (APC), and T-cells are the three major players involved in this process.

Proteins encoded in the MHC are the major antigens that stimulate graft rejection. These MHC antigens may be MHC class I or class II. Class I MHC antigens are expressed on the surface of all nucleated cells. Class II MHC antigens are mainly expressed on the cell surfaces of B -lymphocytes, macrophages, and dendritic cells, which also are known as APCs. The main function of APCs is to present antigens to T-cells. In addition to this, the APCs also transport the antigens to lymphnodes where naive T cells are located, facilitate the binding of T cells, and provide stimulatory signals for T-cell activation.

The activation of the recipient´s T-cells can occur by three distinct pathways of allorecognition: direct, indirect and semi-direct pathways. Direct allo-recognition is the interaction of T-cells of the recipients through the T-cell receptor (TCR) with the allogeneic MHC molecules presented by graft-derived APCs, and in particular by dendritic cell. When peptide derived from donor MHC antigens are processed and present by recipients APCs, this process is known as indirect pathways. Semi-direct allo-recognition is the pathway wherein recipient APCs acquire donor MHC through cell-to-cell contact, which activates a T-cell response in the recipient.

When the MHC-antigen complex on APC is bound by receptors on the naïve T-cell, antigen-specific signals get delivered to the T-cell through the TCR-CD3. These signals are not sufficient to activate T cells. A second essential signal is costimulatory molecules that are engaged with their ligands on APCs. The interaction of CD28 on the T cell surface with its APC surface ligands, B7-1 or B7-2, is one of the major costimulatory pathways. Additional costimulatory molecules include the CD40 and its ligand CD40L (CD154). Once the T cells are activated, they become effector T-cells and mediate allograft rejection.

Chronic rejection

Chronic rejection occurs within month or years after transplantation and gradually causes a continuing weakening of graft function. Although there are differences in appearance of chronic rejection between organs, it is usually characterized by fibrosis of the graft and associated vasculature besides progressive deterioration of graft function. It is estimated that chronic rejection affects up to 50% of allografts after five years of transplantation (48). Thus, chronic rejection remains a serious obstacle in solid-organ transplantation.

Immunosuppression

Immunosuppressive protocols in the clinical setting can be categorized as either induction/ maintenance therapies to prevent allograft rejection or short courses of intensive therapies, also known as rescue therapies, to suppress an acute episode of rejection. Different pharmacological agents that are used in organ transplantation and their mode of action are presented in Table 1.

Induction therapy works by depleting the circulating T lymphocytes of the recipient, and will then delay the onset and severity of the first episode of acute rejection. The induction therapy currently used in solid organ transplantation usually consist of high dose of maintainance drugs (calcineurin inhibitors, corticosteroids) and also includes polyclonal antithymocyte globulins (ATG), anti-interleukin-2 (IL-2) receptor monoclonal antibodies, such as daclizumab and basiliximab, Campath-1H (alemtuzumab), and anti-CD3 monoclonal antibodies..

The maintenance therapy is typically designed as a tri-agent approach. The most commonly used immunosuppressive combination is that of a calcineurin inhibitor (tacrolimus or cyclosporine) an antiproliferative agent, such as mycophenolate mofetil, and corticosteroids, such as prednisolone.

Treatment or “rescue” therapy is provided during episodes of acute rejection and typically starts with corticosteroid boluses.

Table 1 Common immunosuppressive agents used in organ transplantation

Drug	Mode of action
Induction	
Anti-thymocyte globulins	Reduces number of effector T cells, and blocks their function
IL-2 receptor antagonists (daclizumab, basiliximab)	Targets IL-2 receptors on T cells and block their IL-2-dependent activation
Maintenance	
Cyclosporine	Inhibitor of T cell activation, binds to cyclophilin and this complex inhibits calcineurin phosphatase and thus inhibit IL-2 production and T-cell activation
Tacrolimus	Inhibitor of T cell activation, bind to FKBP12 to inhibit calcineurin phosphatase and thus inhibits IL-2 production and T-cell activation
Prednisone, prednisolone, methyl prednisolone	Multiple anti-inflammatory and immunomodulatory effects, Inhibit the transcription and production of several pro-inflammatory cytokines Inhibit macrophage activation
Azathioprine	Converts 6-mercaptopurine to block the de novo pathway of purine synthesis by formation of thio-inosinic acid
Mycophenolate mofetil	Blocks purine synthesis, inhibits proliferation of T and B-cells
Sirolimus Everolimus	Inhibits interleukin-2 driven T-cell proliferation

Aims of the study

The general aim of this thesis was to develop an animal model for UTx in the rat and to use this to explore various issues on UTx.

The specific aims were:

1. To develop a model for heterotopic UTx with comparisons to a native uterus (*Paper I*)

2. To further develop the vascular UTx model in the rat to enable spontaneous mating and to assess pregnancy outcome in a syngeneic model *(Paper II*)

3. To establish whether tacrolimus, as a single immunosuppressive agent is able to prevent rejection in an allogeneic UTx model (*Paper III)*

4. To investigate the feasibility of different sites for diagnosis of early rejection in an allogeneic UTx model in the rat *(Paper IV)*

5. To determine if allogeneic UTx in the rat with immunosuppression is compatible with fertility *(Paper V)*

6. To determine if offspring from an allogeneic UTx situation is of normal birth weight and demonstrate normal growth trajectory (*Paper VI*)

Materials and Methods

The methods, animals and materials that have been used in the uterus transplantation (UTx) studies of this thesis are briefly described below. Detailed descriptions can be found in the "Materials and Methods" sections of individual papers.

Animals and experimental groups

Adult, virgin female rats that were used as uterus donors and recipients in all UTx studies weighed between 150 and 170g. Male Lewis (Paper II) or Sprague Dawley rats (Papers V, VI) of proven fertility were used for mating. Rats that were used in Paper I, II and III were purchased from Charles River, Sulzfelt, Germany. Rats that were used in Paper IV, V and VI were from Harlan Laboratories, Horst, Netherlands.

After arrival from the breeder, all the animals were housed in the animal facility of Experimental Biomedicine of the University of Gothenburg. The housing conditions were 22ºC with controlled light/dark cycles of 12h: 12h and with free access to pelleted food and water. All experimental protocols were approved by the Animal Ethics Committee in Gothenburg, Sweden.

In Paper I and II, transplantation was performed between genetically identical animals of the same inbred strain (syngeneic transplantation). In Paper III –VI, UTx was done in allogeneic setting, which was transplantation between different inbred strains of rats. A summary of all rat strains used and the type of UTx is given in Table 2.

Control groups were included in several studies. Groups with sham-surgery were included in Paper II, III, V, and VI. Groups with administration of immunosuppression but not undergoing UTx were included in Paper III, V and VI. The native uterus was used as control tissue in Paper I and IV.

Table 2 Rat strains and type of UTx in experiments of Paper I-VI

Paper	Donor	Recipient	Transplantation model
I	Lewis	Lewis	syngeneic, heterotopic
II	Lewis	Lewis	syngeneic, orthotopic
III	Brown Norway	Lewis	allogeneic, orthotopic
IV	Lewis	PVG	allogeneic, orthotopic
V	Dark Agouti	Lewis	allogeneic, orthotopic
VI	Lewis	PVG	allogeneic, orthotopic

The experiments involving UTx were performed utilizing four different combination inbred rat strains: Lewis ($RT1^{l}$), Brown-Norway ($RT1^{n}$), Dark Agouti (DA, $RT\text{-}1^{a}$) and PVG (Piebald Virol Glaxo, $RT1^{c}$). All of these strains have been widely used in transplantation-related research for many years.

Lewis ($RT1^{l}$) rat is an inbred stain of laboratory rat, developed from Wistar stock in the early 1950s. This strain was used in experiments of Papers I and II, since these rats show high fertility and have pelvic vascular anatomy that is favourable for UTx surgery.

The Brown-Norway ($RT1^{n}$) is also an inbred stain of laboratory rat, but the pelvic vascular anatomy may differ between animals of the same litters. Brown-Norway ($RT1^{n}$) to Lewis is a commonly used rat strain combination in experimental multivisceral and intestinal transplantation (49). This rat strain combination differs at both major and minor histocompatibility loci (50) and has been found to produce complete allograft rejection after experimental lung (51) or intestinal (52) transplantation, when immunosuppression is not given. This combination of rats was used in the study III.

In Paper V, the combination of Dark Agouti (DA, $RT\text{-}1^{a}$) as uterus donors and Lewis (LEW) was used, which is also a fully allogeneic model (53), with fulminant transplant rejection if immunosuppression is not used (53, 54).

The Lewis to PVG combination was used in tests of fertility (Paper VI) since this allogeneic combination may induce a slightly weaker immune response as shown in

experimental intestinal transplantation (55). Since the most likely event in future human UTx would be a reasonable but not full MHC match, this combination was used in Paper VI. However, preparatory studies with UTx and no immunosuppression given showed complete rejection with this strain combination.

Anaesthesia

The animals were anaesthetized using isoflurane (5% isoflurane was used for induction and 1-1.5% isoflurane was used for maintenance of anesthesia) in a mixture of air (600ml/min for induction, 300 ml/min for maintenance) and oxygen (600 ml/min for induction, 300 ml/min for maintenance). The operations were performed under an operating microscope (Leica M651; Leica Microsystems, Wetzlar, Germany). Throughout the surgery the body temperature was maintained at 37^{o} C, by keeping the animal on a warm heating pad.

Surgery of uterus retrieval

The procurement of the uterine graft was basically the same in Papers I-VI and the procedure is described in more detail in the individual papers.

In brief, after the rat was anesthetized, heparin (1000 IU/kg body weight) was injected sc and a midline longitudinal abdominal incision (from the sternum to pubic symphysis) was performed to open the abdominal cavity. A graft containing the right uterine horn, the common uterine cavity with the cervix and the upper part of the vagina and a vascular pedicle comprising the right uterine vessels with the ipsilateral internal iliac vessels up to and including the entire common iliac vessel was isolated.

The graft was then flushed in situ with 1-2 ml of cold (4°C) Perfadex preservation solution (Vitrolife AB, Mölndal, Sweden), supplemented with xylocaine (0.4 mg/mL) and heparin (50 IU/mL) until the graft was uniformly pale and the venous effluent was clear. The harvested graft was then kept in Perfadex solution (4°C) while the recipient was prepared for UTx. In Paper I, Ringer Acetate supplemented with xylocaine and heparin was used as both flushing and preservation solution. All experiments involving the donor animal was ended by the donor rat being euthanized by cardiac puncture.

Surgery of the uterus recipients

Heterotopic UTx, with transplantation of the uterus to an unphysiological position, was performed in Paper I and orthotopic UTx, with transplantation to the normal position, was performed in Paper II-VI.

Orthotopic uterus transplantation

In the recipient, a hysterectomy was performed with (Paper II, V and VI) or without (Paper III and IV) preserving a small portion (1cm) of the upper part of the right uterine horn (Fig. 1). This procedure was initiated after injecting low molecular weight heparin (dalteparin; 500UI/kg bodyweight) sc, to prevent thrombosis formation during further surgery.

For vascular anastomosis, the right common iliac vessels were dissected free and separated from each other for the whole length from the aortic bifurcation until the branching of the internal and external vessels. The grafted uterus was brought from the backtable into its position within the abdomen of the recipient, where the uterus was kept cold during the period of anastomosis surgery by intermittent dripping of cold (4°C) physiological saline. Vascular clamps were place over the artery separately and a very small portion of the arterial wall was removed to make a hole on the anterior wall of the right common iliac artery. This place would accommodate anastomosis with the end of common iliac artery of the graft. An end to side anastomosis was performed between the common iliac artery of the graft and recipient´s right common iliac artery, using semicontinuous (Paper II, III, and V) or interrupted (Paper IV and VI) 10-0 nylon suture (Fig. 1). This was followed by a similar procedure for the venous anastomosis using an end-to-side fashion with 10-0 (Paper II, III, V) or 11-0 nylon (Paper IV and VI) hemicontinous suture.

In Paper II, V and VI fertility after spontaneous mating was an endpoint. In these experiments the tip of the uterine horn of the graft was anastomosed with the native uterine tip that was kept at the end of hysterectomy (Fig. 1). In Paper III and IV, the tip of the uterine horn of the graft was fixed with a single 7-0 nylon suture to the tissue surrounding the clip that had been placed during hysterectomy, in a position that was between the tip of right uterine horn and the right oviduct of the recipient.

In all these experiment the vagina of the graft was anastomosed to the vaginal vault with interrupted resorbable 6-0 polyglactin sutures.

The abdomen was subsequently closed in two layers using a continuous 6-0 polyglactin suture in two layers. Three ml of 4% icodextrin solution (Adept ®; Baxter, Deerfield, IL, USA) was placed inside the abdominal cavity before closure to prevent post-operative intra-abdominal adhesions (56) .

Heterotopic uterus transplantation

In Paper I, heterotopic UTx was done and the native uterus of the rat was kept intact (Fig. 1). The vascular anastomosis was prepared between the common iliac vessels of the graft and recipient's aorta and vena cava, using semicontinuous 10-0 nylon suture. The vaginal end of the graft was exteriorized through the abdominal wall to create an abdominal stoma. The tip of the uterine horn remained free in the abdominal cavity. The abdomen was closed as described above.

Sham surgery

Sham-surgery was used in Paper II, III, V and VI to create appropriate control groups. Sham-operated animals underwent laparotomy with removal of left uterine horn to achieve an animal, which was anatomically similar as after UTx. Via midline laparotomy a titanium clip was placed between the left oviduct and the tip of the horn and another clip was placed between the proximal edge of the left uterine horn and the common uterine cavity. The portion of the uterus between the two clips was removed and the abdomen was closed.

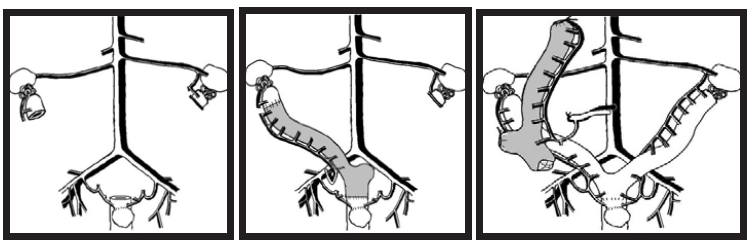

Figure 1. Schematic drawings of UTx procedure. **Left panel**; Hysterectomy in orthotopic uterus transplantation procedure. **Middle panel**; Orthotopic placement of an uterus. **Right panel;** Heterotopic placement of an uterus. The grafted uterus is shown in gray.

Immunosuppression

In Paper III, V and VI immunosuppression by tacrolimus was used to prevent rejection of the allogeneic uterine grafts. The rats that received immunosuppression were given tacrolimus sc via implanted Alzet 2ML4 or 2004 model miniosmotic pumps (Alzet, Durec, Cupertino, CA, USA) at a dose of 0.5 or 0.4 or 0.2 mg/kg/day based on the study protocol. The pump was filled with a solution of tacrolimus (tacrolimus diluted in 0.9% NaCl), which was prepared individually for each animal in relation to the weight of the

animal and then primed in 0.9% NaCl solution at 37°C for 24 h according to the manufacturer's instructions. Then, at the end of surgery in the UTx animals or as a single procedure in the control groups, the pump was implanted in the sc. space on the back of the animal. This procedure was performed under isoflurane anesthesia and the wound was closed with Micelle clips (Kent Scientific, Torrington, CT, USA).

Tail blood samples were collected throughout the experimental period and blood concentrations of tacrolimus were measured by a chemiluminescent immunoassay (ARCHITECT®, Abbott Scandinavia AB, Solna, Sweden).

Reproductive performance after uterus transplantation

In Paper II, V and VI, the endpoinf of these experiments was pregnancy. After a recovery and healing period of six to eight weeks after surgery (UTx or sham-surgery), the female rats were placed with males of proven fertility. After that, the female rats were examined every morning for 4 days for an indication of mating. Those with presence of vaginal plug were considered to be pregnant (defined as Day 0 of pregnancy). Based on the study protocol, delivery of the litters or the rat was allowed to go vaginally (Paper II) or planned cesarean section was done on either pregnancy Day 17 (Paper V) or Day 22 (Paper VI). The litter size and the number of living pups were noted. At the end of the cesarean section or delivery, all animals were euthanized and their uteri were examined for signs of partially resorbed pregnancies and uterine biopsy was collected.

The pups were then placed with foster mothers. Sprague-Dawley females delivered per vaginally 1 or 2 day prior to the day of the cesarean section of experimental animals were used as foster mothers. The pups were then followed by weekly weight measurements from Day 1. The follow-up period ranged from 6 weeks (Paper II) to 19 weeks (100 days, Paper VI) according to the study protocol.

Graft harvesting

Depending on the study protocol (Paper I, III and IV) the uterine tissues were collected on the pre-defined post-operative day. Each harvested uterine graft was cut into three portions. Portions for histological staining and immunohistochemistry were fixed in 4% normal buffered formalin solution. Sample portions for RNA isolation were placed into RNA later and then were stored at -20°C.

Histology

Detailed histological analysis of uterine grafts was performed in Paper I, III and IV. Tissue biopsies from the grafted uteri were collected and submerged into buffered

formaldehyde (4%). This was followed by dehydration and the specimen was later embedded in paraffin and sectioned (4 µm thick). The sections were then stained with eosin and hematoxylin and analyzed under light microscopy.

Immunohistochemistry

Immunohistochemistry was used in Paper III to detect and quantitate CD3, CD4 and CD8 positive lymphocytes. Rabbit polyclonal anti-CD3 (Abcam, Cambridge, UK), mouse monoclonal anti-CD4, (Serotec, Düsseldorf, Germany) and mouse monoclonal anti-CD8 (Serotec) were used as primary antibodies.

In the protocol, deparaffinized slides were rehydrated through descending ethanol concentration and then boiled in a pressure cooker with 0.01 M citrate buffer with 0.05% Tween 20 (pH 6.0) for 20 min or treated with pronase for antigen retrieval. Following washing in Tris-buffered saline (TBS), the sections were incubated with Biocare's Background Sniper (Biocare, Concord, CA, USA) for 10 min at room temperature to block non-specific staining. Primary antibodies against the T-lymphocyte specific antigens CD3, CD4 and CD8 were subsequently added and kept overnight at 4°C. After washing with TBS for 15 min, MACH 3 Mouse AP-Polymer detection kit (Biocare) was applied to the sections according to the manufacturer´s instructions. Slides were rinsed in TBS and then incubated with Vulcan Fast Red (Biocare). Finally the sections were counterstained with hematoxylin.

The numbers of positively stained cells were counted within a grid (10 × 10 squares, 0.125 mm^2 in ×40 objective and ×10 eyepieces) by two independent observers (blinded fashion). In each section, five randomly chosen areas each of the endometrium and the myometrium were counted and the average values for each section were used as individual data points.

Quantification of mRNA

In Paper III, messenger ribonucleic acid (mRNA) levels of certain genes were quantified. At first, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) from the uterine tissue sample according to the manufacturer´s protocol. The integrity of the extracted mRNA was then assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Kista, Sweden).

Extracted mRNA was then reversed transcribed into complementary deoxyribonucleic acid (cDNA) using 2 µg total RNA with 0.5 µg random primer (Promega, Madison, WI, USA), 0.5 mM deoxy-NTP, 20 U RNAsine, RT-buffer and 200U Moloney murine leukaemia virus reverse transcriptase (Promega).

Real-time quantitative PCR (rtPCR) in an ABI Prism 7000 Sequence Detector system (Applied Biosystems, Foster City, CA, USA) was performed to evaluate the mRNA expression. Commercially available Taqman MGB probes (Applied Biosystems) were used for the target genes. Each amplification reaction consisted of 20 ng cDNA, 1x probe-mix and 1x TaqMan Universal PCR mastermix (Applied Biosystems) to a final volume of 25 µl. After control of amplification efficiency of the targets genes and the control genes, the relative expressions of mRNA were calculated using the comparative C_T method (57). Expression of the target gene mRNA were normalized to the expression of the control (β-actin).The mRNAs for interleukin (IL)-1α, nuclear factor (NF)-κB, interferon-inducible protein (IP) 10, leukemia inhibitory factor (LIF), galectin-1, CD200 and IL-15 were studied in Paper III.

Statistics

In Paper I, IV and V, no statistical analysis was done. Concerning Paper I, data related to surgical time and survival is presented as mean ± standard deviations of mean and data related to quantification of neutrophils presented as medians with 10-90% ranges. Concerning Paper IV and V, data related to surgery, cold and warm ischemic times are presented as medians, ranges, and pregnancy rate is presented as percentages (%). The non-parameteric Wilcoxon signed-ranks test with presentation of data as medians and ranges was used to compare durations of surgery and cold ischemia (Paper II), as a post-test after Kruskal Wallis test for data of Paper III. For multiple comparisons the nonparametric Kruskal Wallis test (Paper III, VI) or the parametric ANOVA test (Paper VI) were used.

Mating, pregnancy and conception frequency between transplanted and control animals of Paper II were compared using X^2 test. For all comparison, a p-value of <0.05 was considered to be statistically significant.

Results and Comments

Paper I

Background

Intense research in the field of UTx has been ongoing since around year 2000. This scientific activity started after the first human UTx case was performed that year and later published in year 2002 (58). The first animal UTx-model, which was developed and also properly explored, was the mouse model. The research group that I belong to showed for the first time pregnancies after syngeneic mouse UTx (59) and later also normal offspring in this model (60). Other groups around the world investigated large animal models such as the pig (6, 61) and the sheep model (62) in scientific efforts to move the field towards human UTx.

A drawback with large animals is of course the large costs of the animals and the complicated and personal-intense animal care as well as that there is a shortage of inbred strains of large experimental animal, which can be used for syngeneic transplantation experiments. These syngeneic UTx models are of great use since there is no rejection, and the outcome can be interpreted as a result mostly secondary to surgery or ischemia reperfusion events.

In the field of transplantation, the rodent models of the rat and the mouse are most frequently used and are in many ways suitable first line transplantation research model, due to the comparative easy handling. Moreover, preoperative and post-operative care of small animals is easier than large animals. The rat may be a more suitable model than the mouse because of several reasons. This assumption is based on that microsurgery in mouse model is very complicated (59) (63) due to small size of the blood vessels and pelvic organs, which is related to the body weight of the mouse being around about 25 g. Furthermore, the relative doses of immunosuppression that is needed to prevent rejection of allografts in rat are in agreement with that of the human (64, 65). Thus, in attempts to prevent rejection of allografted mouse uteri it was found that very high levels of cyclosporine had to be used and that the results was more that the rejection was delayed somewhat, rather than prevention of rejection (66). Similarly, cardiac allografts in a mouse model were found to be rejected when CsA trough levels reached 1000 ng/ml, in contrast to the rat model in which only one-tenth of that dose of CsA prevented heart rejection (67).

In the present study the rat was explored for the first time as a model for UTx. The methodology was based partly on the technique from the previously published mouse model (60) but modifications were performed to adjust for the larger vascular anatomy of

the rat. In the first set of experiments inbred Lewis rats were used both as donors and recipients, with the weight being around 5-6-fold larger than mouse model.

Results

The donor operation was developed to isolate the right uterine horn and the common uterine body and the cervix with a small vaginal rim. The left uterine horn was excised in a way that only blood vessels from one side needed to be included in the preparation. The hypothesis was that the ipsilateral uterine blood vessels would be connected to the remaining uterine horn but with the descending parts of the uterine artery/vein being sufficient for perfusion also of the common uterine body, the cervix and the upper part of the vagina.

In the recipient, the subrenal aorta and vena cava were prepared for anastomosis. The common iliac artery and vein of the graft was then sutured end-to-side to the subrenal aorta and subrenal vena cava. The vaginal rim of the graft was exteriorized through a stoma, which was created on the right side of the laparotomy scar.

Two previously untrained microsurgeons were compared. Concerning the factor surviving animals, the rate was around 50-70% in the first animal but increased up to 100% in animals after number 30. The number of viable transplants in surviving animals was fairly constant and similar between the two microsurgeons with around 80% surviving grafts. Naturally the duration of surgery in both the donor rat and the recipient rat decreased with time, so that at the end of the experimental the total surgical time for the recipient was about 160 $\pm$15 min and donor surgery took 70 $\pm$ 20 min.

Concerning both investigators, the surgery of the recipient animal, including both dissection of the large blood vessels, as well as anastomosis of the vasculature and exteriorization of the distal end of the graft through the abdominal wall, was substantially longer than the recovery surgery of the donor animal. Both morphology and histology of the transplants carries out shortly after transplantation showed that the grafts were swollen and somewhat darker in colour than the native uterus. Moreover, a small accumulation of inflammatory cells was found in the uterine tissue at this immediate post-transplantation period. On day 21 after surgery, both the gross morphology and the histology were similar to that of the native uterus.

Comments

This study represents the first detailed study on a rat model for UTx. One important aspect is that the surgery in this rat UTx model seemed to be manageable also for previously unskilled surgeons. Both surgeons of the present study did not have any

previous microsurgical skills or any general surgical training. As expected, there was a typical learning curve, but the survival of the animals was almost 100% already after 30 animals. It should be noted that the graft survival was fairly constant and concerning one of the surgeons, it was already high at rat 1-10 and decreased from 80% to 40% in rat 21-30, to show an increase after that. This biphasic pattern is not typical for any surgical learning curve. It could reflect that the surgeon at this initial stage had overconfidence with the surgery, but with experience awareness of several surgical obstacles may have affected the results. Another explanation could be that the effort to speed up surgery negatively affected the outcome.

The other investigator showed more steady increase in graft survival. In the last cohort of animals, when both surgeons had graft survival of more than 70%, some animals were investigated on postoperative day day 1, 7 and 21.

The investigation of the uterine graft involved gross morphology, histology and estimated the number of infiltrating neutrophils. The fact that the uterine transplants were slightly darker and appeared somewhat more swollen than the native uterus at day 1, probably reflect the tissue response to the surgical trauma and the ischemia of the uterus. This would be expected also in a normal surgical situation, where only gentle manipulation of the uterus is performed. Concerning the ovary, it has been shown that even if the ovary is only slightly touched during surgery, there will be focal loss of surface epithelium (68). Similar mechanisms are most likely also operative concerning the serosal surface of the uterus.

At day 7 and 21 of surgery, the gross morphology of the transplanted uterus was similar to the native uteri. Histology showed small signs of inflammation in the day 1 transplants but the difference between the grafts and the native uteri disappeared during the follow up time. The number of neutrophils was in the same range in the native and in the graft uteri during the entire study period. Thus, no firm conclusion could be done whether any minor inflammation persisted in the transplanted graft.

During the last decade several animal models for UTx research have been developed.

According to the large animal models the rat will of course have the drawback of its small size and that this would necessitate surgical skills by the investigator. Importantly in the present study we could show that two previously inexperienced microsurgeons with no previous training in clinical surgery were able to master this method after a relatively short learning period. It should be noted though that the grafts survival did never reach above 80% apart from early success of one of the investigators. It may well be that the graft survival would reach even higher with longer learning.

However, it is my experience after doing more than 250 surgeries in a similar animal model that there will always be some losses of organs and that this is usually due to formation of thrombosis. Stenosis on the venous side or inadequate handling of graft during surgery is the most likely reason of thrombosis. Thus, the venous anastomotic size should also be made as wide as possible without any twist. Furthermore, the length of the graft artery and vein should be similar. One more important point to reduce graft loss is that the graft should be covered with a wet gauge and cold saline should be poured intermittently over the graft during the period of anastomosis to keep the graft cold. Minimum manipulation of the graft as well as graft vasculature is also crucial to reduced graft thrombosis.

When removing blood from the graft uterus, aggressive flushing should be avoided. Moreover during recipient preparation, while the graft is preserved in cold preservation solution and it should be immersed fully during the entire period.
It should also be stated that, during the study period of this thesis a significant number of animals died between post-operative day 1 and 2. Bleeding from the anastomosis site was the main reason of death of these animals. Bleeding from venous anastomosis can be minimize by meticulous provition of hemostasis matrix but bleeding from the arterial anastomotis can rarely be stopped using hemostasis matrix. Moreover it causes extensive adhesion. Thus, I favour to use an extra suture using 11-0 nylon at the site of bleeding in both arterial and venous anastomosis rather than using meticulous hemostasis.

In clinical transplantation there will also be a very small but significant loss of grafts due to surgical reasons. However the difference is that even if thrombosis occurs early on in post transplantation this can usually be corrected by a re-surgery within a short time.

In the present study we had none who was observing the post transplantation blood flow in the graft and could not do intervention surgery. Both gross morphology and light microscopy were used to evaluate the graft function. In this case, naturally light microscopy is a more sensitive method but the results indicated that the findings go in parallel so that when a slight inflammation was present in the tissue this was paralleled by also slight gross morphology signs.

Several histological methods can be used to assess the grade of inflammation. In the present study, the number of neutrophils was used as a measure to assess the grade of inflammation. It is known that extensive inflammation with primarily influx of neutrophils occur after ischemia re-perfusion injury to most transplanted organs (69, 70) injury and this is the reason why this parameter was assessed. We have previously shown that the human uterus is comparable resistant to cold ischemia (71), since spontaneous and prostaglandin-induced myometrial contractions were seen after 24h of cold ischemic preservation. In a recently accepted study on warm ischemia tolerance of the transplanted

rat uterus we also show the uterus is fairly resistant to warm ischemia, with warm ischemic times of 3-4 h (72).

A small number of the assessment of uteri on day 7 and day 21 had swollen uterine lumina with obstructive cervical opening. This is probably due to that the surgery of the stoma was not optimal in that sense that the opening was constricted. This of course needs further development in the future experiments on a heterotopic UTx model.

Paper II

Background

The reason to carry out research and development concerning UTx is of course to perform this procedure as one way to find a treatment for women with absolute uterine factor infertility. The women with absolute uterine factor infertility, either lack uterus or have a non- functioning uterus, which is unable to implant an embryo or is not functional to nourish the growth of an embryo to a pregnancy length, which allows for delivery of a healthy baby. . Other future alternatives apart from UTx, although today relatively uncertain, for absolute uterine factor treatment could be development of a bioengineered uterus, created by stem cells on a synthetic matrix, as performed concerning the human trachea (73), or to use decellularized tissue from multi-organ donors. This latter technique, with decellularised tissue recomposed with autologous stemcells, was lately successfully carried out to form a vein graft, which was then successfully transplanted into a 10 year old girl who was suffering from extrahepatic portal vein obstruction (74). Both these future alternatives would mean that the patient would not need any immunosuppression, since the stem cells would be autologous.

Another future possibility, which has been discussed concerning the uterus, is that the whole pregnancy can be carried out outside the body, in an artificial incubator which would mimic the in vivo situation inside the uterus (75).This so called ectogenesis approach has been partly successful in goats, where fetuses were removed from their dams and then supported with an artificial placenta and uterus for 3 weeks (76).

The functionality of a transplanted uterus can of course be examined by different means. In the short time-course, assessment of functionality would incorporate normal and viable histology of the specimen. After that, a normal cyclic pattern of the uterus would indicate that the uterus reacts to the hormonal changes during the cycle, and that it most likely would carry an endometrium that is prepared for implantation. However, since the ultimate goal of UTx is to treat infertility of the patient group with absolute uterine factor infertility, the definitive functional test is of course examination whether a transplanted uterus can carry a pregnancy to term and to deliver live offspring.

In a clinical human situation, allogeneic uterus transplantation would be used, rather than the syngeneic transplantation setting, which had been used previously in the mouse (59, 60) and also in the rat in the present paper. Occurrence of pregnancy after allogeneic UTx may be prevented both by factors due to the surgical trauma of transplantation, the new blood flow of the uterus or possibly by immunosuppression to prevent rejection. It would thus be very difficult to interpret the data on tests of pregnancy potential in an allogeneic setting since this involves many unknown factors. The present study was developed as an initial step towards allogeneic UTx in the rat. Thus, syngeneic UTx was used with fertility assessment after spontaneous mating.

Results

The transplantation surgery was modified from that of Paper I so that the anastomosis of the vasculature was performed using the right-sided common iliac vessels of both of the graft and of the recipient. The anastomosis was carried out end-to-side using 10-0 sutures and all of the surgery was performed under a steromicroscope.

In order to allow for pregnancy after spontaneous mating, the previous rat UTx method (Paper I) was also modified apart from the vascular anastomosis, so that the uterus was connected to the vagina and that undisturbed transport of sperms up to the oviductal-uterus junction would still be possible. Thus, instead of having the previously heterotopic position of uterus, the recipient was hysterectomized but leaving both ovaries intact and also a very short tip of the native uterine horns. The graft (right uterine horn, common uterine cavity, cervix with vaginal rim) was placed in an orthotopic position with anastomosis of the tip of the right horn of the uterus graft with the minute tip of the right uterine horn of the native uterus. The vaginal tissue of the graft was also sutured end-to-end to the native uterus of the recipient. Control animals also underwent surgery so that the left uterine horn was surgically excised in the same manner as it was done on the uterus to be transplanted, and in that way both experimental groups contained uteri with only one horn.

The total success rate was 70%. Some animals did not survive the surgery due to bleeding or other reasons and furthermore a small number of animals did not have a satisfactory graft at second-look laparotomy, which was done in all cases 2 weeks after surgery. The cold ischemic time was around 2 h and the surgery time of the recipient was somewhat longer than 3 h. This surgical duration was considerably higher than the surgical time of around 40 minutes of the sham group.

The 19 animals of the uterus transplantation group were compared with 19 animals of the sham group. Out of the 19 transplanted animals, 17 mated during the first cycle and 11 became pregnant. The pups per pregnancy were about 3 in each group. The number of

resorbed pregnancies was higher in the uterus transplantation group. The weight trajectory for the pups born from the UTx rats was similar to the pups of control rats and these offspring were normal up to adult age.

Comments

This study represents the first published study on pregnancy in and live offspring from a truly transplanted uterus as a result of spontaneous mating. My research group has previously shown live offspring after autologous UTx in the sheep (77), but those results are not comparable to these since in the sheep experiments of the sheep the uterus was not moved between different individuals. It should also be mentioned that pregnancies have never been reported in a rat UTx model prior to this study.

The choice of animal model for this study was based on that several investigators in the group had difficulties in obtaining success with the heterotopic UTx model in the mouse, as was initially used by the group (59). In the mouse, also orthotopic UTx was tested but with no pregnancies occurring after embryo transfer (Randa R Akouri, personal communication), which is in contrast to the heterotopic UTx mouse model with reasonable pregnancy rate (59, 60).

The advantages of an orthotopic UTx model, as developed in the present study, is naturally that the uterus is in its usual anatomical location, and this may be important for its cyclic physiological changes but also to allow for expansion at pregnancy.

The results of the present study show a reasonable pregnancy rate after orthotopic UTx, but there are several factors apart from the UTx that is not controlled for and that can affect the results. Thus, it may be that the extended surgical time of the UTx group, as compared to the control group, could have affected the ovarian function so that less numbers of oocytes would be released at ovulation.

Concerning the end result of the inner reproductive tract after UTx, both the vaginal-vaginal anastomosis and the utero-utero anastomosis may negatively influence the transport of sperms and embryos. It may also be that the blood flow of the oviduct is impaired after UTx, since the blood flow coming from the uterus would cease due to the surgical procedure.

Even though the pregnancy rate of the experimental group was comparable to that of the control group, only few live births were accomplished from the experimental group. One can speculate that this was secondary to denervation of the uterus at transplantation and that this would affect the mechanisms of labour. Another explanation is that there was

some constriction in the vaginal anastomosis, although sutured with resorbable sutures, and that this would prolong the labour and thereby lead to fetal asphyxia.
Of note is that the vascular anastomoses were accomplished by nylon sutures, which do not show any resorption in vivo. Thus, this would not allow for any expansion of lumen at the anastomosis site lumen during pregnancy, when the uterine blood flow is considerably increased.

Paper III

Background
The magnitudes of the immune response and rejection vary with the type of tissue or organ that is transplanted. It is well described in transplantation surgery that the transplanted liver does usually not require heavy immunosuppression and some of these transplanted patients can be taken off completely from immunosuppressive therapy some years after transplantation. Thus, weaning of liver transplant recipients from their immunosuppressive drugs is widely practiced and several reports on liver recipients have shown that a liver allograft can maintain normal function without immunosuppression. Although drug withdrawals in these studies were associated with rejection in some recipients, the majority of these episodes of rejection was mild or was easily resolved (78-80). In contrast, vascularized composite allografts, such as the hand and face, as well as intestinal transplants, usually require high doses of immunosuppression and also combinations of several drugs to avoid full rejection. This is thought to be due to that these tissues/organs contain large population of immunologic active cells such as the Langerhans cells of the skin and lymphoid tissue within the intestine.

There exist only few studies concerning what immunosuppression to use after UTx in an allogeneic setting. In initial experiments from the early 1970s with the uterus transplanted by an avascular approach in the dog (81), the uterus was wrapped inside the omentum and the blood flow was re-established by neoangiogenesis. Azathioprine and corticosteroids were used as immunosuppressants but patchy necrosis was seen within one week and the allo-transplants later became fully necrotic.

During the early 1980s, the first calcineurin inhibitor was introduced as a potent immunosuppressive agent. This first calcineurin inhibitor was cyclosporine A, which is a substance that was purified from either of two different fungi. Its main action is to prevent synthesis of interleukin (IL)-2 and thereby to inhibit activation and proliferation of mature T-cells, both of the CD4 and CD8 lineages. When cyclosporine was introduced in the clinic, it resulted in considerable improvement in the success rates concerning graft survival and the calcineurin inhibitors are today the mainstay in the immunosuppression.

Cyclosporine was used as an immunosuppressant in a rabbit model of non-vascular UTx, where the uterus was transplanted into the broad ligament (82). The drug was given by im administration at 15 mg/kg daily for two weeks and then the dose was tapered to 5 mg/kg day. The blood concentrations were not specified. This allogeneic transplantation was carried out in six animals, of which three rabbits developed peritonitis and pelvic abscesses. Three uterine grafts showed well-preserved endometrium and myometrium and the longest observation time was 1 month. Notably is that one of these animals conceived in the non-transplanted uterine horn at a time after UTx. The possible effect of cyclosporine in preventing rejection of a uterine allograft was also examined in a mouse model of UTx (83). In that study, a semi-allogeneic transplantation situation existed with the uterus donors being of C57BL/6 strain and the recipients being F1 hybrids of that strain combined with the CBA/ca strain. The uterus was heterotopically transplanted, with the vascular anastomosis sites being on the aorta and vena cava. Cyclosporine was administered through mini-osmotic pumps that were placed sc at dorsolateral aspect of the thorax. The cysclosporine dose of 10 mg/kg per day resulted in blood concentrations of around 200 ng/ml, while the high dose of 20 mg/kg per day gave a wider variation of blood levels, ranging from around 300 to over 1000 ng/ml. Although the doses cyclosporine used in that study were high, they were unable to prevent rejection-associated changes of the graft at assessment 10 days after transplantation in that mouse model. Thus, normal gross morphology was evident but histology revealed a moderate increase in infiltrating leukocytes. Our laboratory has also conducted one study with cyclosporine as monotherapy in the rat allogeneic UTx model (84). In that study, cyclosporine was administered by daily sc injections of 10 mg/kg and the follow up time was 7 days post UTx. The gross morphological examination indicated some rejection, since a slight uterine enlargement was seen in some animals. Histology showed focal degenerative changes within the endometrium and also scattered lymphocytes in the myometrium and it was concluded that cyclosporine could suppress rejection to a large degree.

Around 10 years ago, tacrolimus was introduced as the second clinically used calcineurin inhibitor in transplantation surgery. This substance is extracted from the fungus streptomyces tsukubaensis and its action is bind to specific FKBP12 receptors and thereby preventing activation of synthesis of IL-2, IL-3, interferon (IFN) gamma and also some other cytokines. Tacrolimus is now used more and more in clinical transplantation. Thus, the most common combination of immunosuppression used in transplantation is today tacrolimus together with mycophenolate and prednisolone.

In the present study it was tested whether immuosuppression by tacrolimus as a monotherapy could inhibit uterine rejection in an allogeneic UTx model in the rat. The study was carried out with Brown Norway rats as uterus donors and Lewis rats as uterus recipients. This combination of organ donor and organ recipient represents a fully

allogeneic transplantation situation which would be similar as transplantation between unrelated humans. There were two sham-groups included in the study as control groups. One sham-group was medicated with tacrolimus but did not undergo surgery and the other group did only go through laparotomic surgery, which was designed to in some way mimic the UTx surgery. All the 4 groups had uteri of the Brown Norway strain but in the sham-groups as the native uterus and in the two UTx groups, as the grafted uterus. Surgery was conducted with isolation of the common part of the uterus and one uterus horn on a vascular pedicle and with transplanting this to the recipient after hysterectomy in that animal. Vascular anastomosis was on the common iliac arteries and veins. The results were evaluated by histology, immunohistochemistry and by measuring expression levels of several mediators that are related to inflammation/rejection and/or implantation.

Results

In this study, a total of 14 animals had to be used to achieve 12 transplanted animals with six of those belonging to the tacrolimus-treated group and six to the non-treated group. Tacrolimus was administered by mini-osmotic pumps at a dose of 0.5 mg/kg per day and this resulted in blood concentrations of the range between 1.2 and 6.3 ng/ml. The viability of the uterus and the histology as well as mRNA-levels of mediators were evaluated 14 days after surgery.

Uterine allografts not receiving the immunosuppression showed clear macroscopic signs of rejection. This was also confirmed in light microscopy, where wide-spread necrosis with only a brim of remaining muscular tissue was seen. The T-cell contents were difficult to assess in the non-treated UTx group because of necrosis. These histological changes differed completely from those of the native uterus of the control groups and the uterine allograft of the tacrolimus-treated group, as further described below. The non-tacrolimus treated transplanted group showed high levels of IL-1 and these levels were higher than in all other groups. Similar results were shown for IP-10. There were lower levels of galectin-1 in the uteri of non-treated transplanted group, as compared to the tacrolimus-treated UTx group and in that of the sham-group treated with tacrolimus.

The gross morphology and light microscopic picture of the uteri of the transplanted group given tacrolimus were similar to the control uteri. Immunohistochemistry showed low numbers of lymphocytes in the native uteri of the sham groups. Similar to the control grafts, allografts in the animals treated with tacrolimus had a very low number of T-cells except in 2 out of 7 transplants, where increased numbers of CD8 positive T-cells were found.

Comments

This is the first study, which has thoroughly investigated the role of the calcineurin inhibitor tacrolimus in the rat UTx model. As stated above, our group has previously investigated whether cyclosporine can be used as a single immunosuppresive agent after UTx both in the mouse (83) and in the rat (84). Those studies found that even very high doses of cyclosporine were unable to effectively suppress uterine rejection in the mouse (83). It should be mentioned that the mouse UTx strain combination was a semi-allogeneic grouping and the rejection potential may not have been full. In the rat (84), the lower of the doses of cyclosporine (10 mg/kg per day) than that was used in the mouse study (6), could almost completely suppress rejection when assessed after 7 days.

In comparison to these studies on cyclosporine after allogeneic UTx, the dose of tacrolimus used in the present study was much lower on a per body weight calculation. This is in accordance with the recommendations of medication to prevent organ rejection after transplantation in the human, with a recommended dose of below 5 mg/kg per day for cyclosporine and doses up to 0.5 mg/kg per day for tacrolimus.

The major finding of the present study was that tacrolimus, as a single immunosuppressive agent and at a low dose, is able to effectively prevent graft rejection in the rat UTx model. This finding may suggest that the uterus is not a highly immunogenic organ or that tacrolimus is especially suitable as an immunosuppressive to prevent rejection of a transplanted rat uterus. In studies of composite tissue allotransplantaion in the rat model (85, 86) it was shown that tacrolimus prolonged graft survival only if it was given at high doses (2-8 mg/kg/day) or if it was used in a combination therapy with other drugs such as 15-deoxypergualin (87).

The analysis of mRNA levels of implantation-related markers included several key mediators. Differential expressions between the groups were seen for three markers. The pro-inflammatory cytokine IL-1 was markedly upregulated in the rejecting allograft and low in the other groups. This result is in accordance with the histology, showing inflammatory and necrotic tissue in the samples. It was reassuring to find that the IL-1 levels in the tacrolimus treated and transplanted group were not elevated. This result is similar to that when cyclosporine was evaluated in the same model but with a shorter (7 days) observation time (84).

In the present study, also IFN gamma-induced protein-10 (IP-10) was evaluated, and this marker had not been tested in any animal model of UTx before. This cytokine is secreted by many cell types in response to stimulation by IFN gamma and it serves many actions including chemattraction for monocytes/macrophages and T-lymphocytes, promotion of T-cell adhesion and regulation of angiogenesis (88, 89). Importantly, this cytokine is also involved in remodulation and regulation of immunecells in the endometrium (90) and it

may thereby be of physiological significance for provision of an endometrium that is optimal for implantation. Implantation is a physiological process that is tightly regulated by the local immune system (91) and it has also been implicated that a small boost of local inflammation at the implantation site may enhance the implantation capacity (92).

The implantation-related marker galectin-1 was also assessed. The levels of mRNA for galectin in the tacrolimus-treated and transplanted group were in the same range as in the control groups, which would indicate that the implantation potential of these animals would be normal.

The results of the study, showing that tacrolimus is able to suppress rejection is promising, towards further studies on fertility potential in the allogeneic rat UTx model. However, it should be mentioned that these studies in the rat could not directly be extrapolated to other species, since certain inter-species differences may exist. We have experienced in our primate model of the baboon that single tacrolimus administration is not enough to prevent graft rejection of the uterus in an allogeneic UTx model (93) .

Paper IV

Background

An important issue in all types of organ or tissue transplantation is to be able to detect rejection at an early stage. The rejection process depends on recognition by the host of the transplanted organ as non-self and several lines of studies indicate that parts of a graft are not uniformly attacked at rejection.

The clinical presentation of rejection is generally divided into the three phases: hyperacute rejection, acute rejection and chronic rejection (94). The hyperacute rejection generally starts within hours after transplantation and is dependent on already formed cytotoxic antibodies that recognize histocompatibility complex 1 or blood group antigens on the endothelial cells of the microvasculature of the organ (45). This will lead to endothelial damage, with secondary thrombosis and blood flow obstruction through the organ.

The dominant form of rejection and what is commonly referred to as organ rejection is termed acute allograft rejection. The first episode of this type of rejection generally occurs within the first 6 months after transplantation, with clinical signs being low grade fever and graft dysfunction. The basis for this acute rejection is recognition by the recipients CD4 T-lymphocytes of non-self antigens on any cell type of the graft. A complex immunological interplay will eventually lead to infiltration of inflammatory cells into the tissue, which in the end can lead to apoptosis and necrosis of organ cells. Thus, it

is of importance to detect these early events of acute rejection so that the rejection process can be reversed.

The third part of graft rejection is the chronic rejection (95), which occurs in most transplanted organs as a very slow but progressive process. The mechnaisms behind this type of rejection is not well understood, but the end result of proliferative lesions in the microvasculature that leads to stenosis, is well described.

As mentioned above, in the events that rejection is detected early and when it is only a mild inflammation, the immunological attack against the grafted non-self tissue can be reversed by modulation of immunosuppression. This is usually accomplished by increasing the doses of the calcineurin inhibitor used in the protocol, increasing cortisone doses or using anti-thymocyte globulin or similar induction therapies for a short while (96). Thus rejection does not mean an irreversible failure of the organ.

In transplantation of organs that faces the exterior of an individual, detection of rejection may be available by biopsy. Thus, skin biopsies could be used for composite allografts such as the hand. Concerning internal organs, such as the kidney, the liver and the heart, these are not easily accessible and invasive biopsy procedures may be needed. An advantage of the uterus is that it is a semi-external organ and that all the parts are inside the body but with the access to the cervix in the upper part of the vagina and to also the endometrial cavity through the external os of the cervix. A small part of the upper part of the vagina may also be from the graft, and this could possibly be available for biopsies.

There have been some studies looking at the uterine rejection after UTx, both by gross morphology and histology (97) as well as by detailed immunohistochemistry to quantiate different leukocyte subtypes (98) in the mouse UTx model. In the latter study, involving uteri from BALB/c mice transplanted into C57Bl/6 recipients, the leukocyte population was investigated at day 2, 5 and 10 after transplantation. The native uterus was kept as an internal control, to be able to compare the leukocyte population of the graft and the native uterus. The main findings were that the neutrophils were increased in the myomerium from day 5 and in the endometrium from day 10. The increase of T-cells followed a similar time-course, while the macrophages were increased already at day 2. Thus, both these studies in the mouse UTx model provide the first important background data concerning the time course of rejection of a transplanted uterus in any animal model. However, these studies only examined the endometrium and myometrium in cross sections of the uterus and concerning the myometrium, this is not a readily available site to acquire tissue biopsies for rejection diagnosis. Moreover, the study indicated that there may be time-related differences between inflammatory influx into the myometrium and the endometrium and this fact may indicate that uterine rejection is not a uniform phenomenon that occurs simultaneously in all compartments of the graft.

In the present study, an allogeneic model with Lewis rats as uterus donors and PVG rats as uterus recipients were used. This strain combination represents a fully allogeneic type of transplantation. It is known that there exist cycle-dependent variations in leukocyte distribution throughout the estrous cycle of the rat (99) but that a pseudopregnant state represents a stage of relative stability regarding leukocyte density. Consequently, the donors and recipients were synchronized by induction of pseudopregnancy to achieve similar distribution of white blood cells. The transplantation was done by an orthotopic approach, where hysterectomy was performed in the recipient and the vascular anastomosis was performed on the common iliacs. Initially, a pilot study of a few animals was conducted to examine when the first morphological signs of rejection was evident in this rat UTx model and it was then decided to include second look laparotomy at either day 4 or 7. Samples were obtained both from the uterus, from the cervix and from the blood vessels to examine different sites. Morphological analysis was done by an expert pathologist with long experience in clinical diagnosis of organ rejection.

Results

The success rate of surgery was somewhat lower than in earlier studies and 25 animals had to be operated to achieve 15 transplanted animals that were used for morphological evaluation of the grafts. The losses of the 10 animals were due to thrombosis or bleeding. The recipient surgery time was around 180 minutes. The morphology of native uteri that was removed at transplantation was used as control. In morphological analysis of the myometrium and endometrium of these specimens, only low numbers of inflammatory cells were present, although in occasional uteri there existed dense infiltrates of eosinophils. In conjunction with these eosinophilic infiltrates, apoptotic figures were seen. Similar findings were seen in the cervical biopsies.

At day 4, the grafts had normal tissue architecture apart from that the endometrial glands showed some degree of dilation and that neutrophils were seen within the glands. Inflammatory cells were abundant within in the blood vessels of the uterine body, but the inflammatory infiltrate was only minor within the stroma of the myometrium. Another distinction from the native controls was that the number of eosinophils was very low in all specimens. In the cervix of the day 4 uteri, the squamos epithelium was found to be thinner and there were also an increased number of inflammatory cells around the glands, which were dilated and filled with neutrophils. At this stage, the vessels showed signs of focal inflammation and in some cases thrombi formations were seen.

At day 7 after transplnatation, the signs of rejection were more general. The uterine architecture was fairly well-preserved but damage to the endometrium was evident by focal loss of luminal endometrium and reduced number of glands. The inflammatory

changes of the uterine body were now evident in all sites with presence of transmural inflammation. In the cervix, the most uniform signs of rejection were evident in the stroma and blood vessels, showing increased number of inflammatory cells. The squamous epithelium of the cervix was in some animals almost normal, while other showed a thinning or a loss of this compartment. The major blood vessels from the graft showed clear signs of subendothelial inflammation and formation of thrombi.

Comments

This is the first study which has thoroughly looked at specific changes at uterus rejection in the rat UTx model. Importantly, the study demonstrates that rejection is a fairly uniform entity with changes seen both in the cervix and in the uterus. Mild inflammation was seen already 4 days after transplantation. At this stage there was no loss of normal tissue and this stage would most likely correspond to a time of early clinical rejection, when increase of anti-rejection drugs could prevent further rejection.

It should be noted that the results of the study indicate that also the cervix is a suitable site to assess rejection. The changes were most stable within the cervical stroma, while the epithelium of the cervix showed an inconstant response. This finding would indicate that in a clinical situation it would not be accurate to determine the rejection state of a transplanted uterus only by ocular inspection of the cervix by the naked eye or by colposcopic examination. In the published human UTx case (58), the authors report that the cervix was dusky-coloured just before removal of the necrotic graft. No details are given on the histological findings of this graft.

This seemingly favourable situation of diagnosis of rejection by a deep cervical biopsy, that includes stroma and intracervical blood vessels, may be important in clinical situations since the cervix is available for biopsies in an outpatient gynecological setting without any need for local anesthesia. Further studies should be done to grade rejection at different sites in other animal models of UTx models in order to understand what the most proper place of biopsies in a clinical setting is. The recent study on allogeneic UTx in the baboon (93), also indicates that the cervix is an appropriate site for attainment of biopsy in order to examine if any rejection reaction is present with the uterine graft.

Paper V

Background

During recent years several studies on UTx have been published. Most of these have examined functionality in terms of presence of viable myometrium and endometrium (62) as well as of menstruation in the cases of non-human primate species (100). Demonstration of pregnancy with live birth is obviously the ultimate goal of UTx. Thus,

pregnancies in syngeneic settings have been accomplished in the mouse (59, 60) and in the rat (Paper II). After autologous transplantation, pregnancies were accomplished in the sheep (77) and just recently also in the cynomolgus macaque (101).

The major question in UTx research is however, if also an allogeneic uterine graft can function in terms of implantation of an embryo and support of an ongoing pregnancy. Such pregnancy would be subjected to several factors that may negatively affect the pregnancy outcome. Some of these factors, such as surgical trauma, altered blood supply, denervation, lack of lymph drainage and ischemia-reperfusion injury would also be present after syngeneic or autologous transplantation. The added factors in the allogeneic setting of UTx are effects of immunosuppressants and possible presence of low grade rejection that is not properly suppressed by the immunoactive drugs.

The present study represents the first attempt in the rat to test whether pregnancies also can occur in an allogeneic UTx situation. Previously, allogeneic UTx had been done before the introduction of calcineurin inhibitors as effective immunosuppressants. In these experiments of avascular UTx in the rhesus macaque (102) and as well as vascular UTx in the dog (103), the immunosuppressants that were used were not able to prevent rejection and the uteri became non-functional and later necrotic. The calcineurin inhibitor cyclosporine was used in a rabbit model of nonvascular UTx but the results of that study was not encouraging (104). Recently, allogeneic UTx with tacrolimus was tested in the rabbit, but with poor survival of the animals (105). The pregnancy potential could not be tested in that study because of the short survival time of the animals.

In the present study, a fully allogeneic model of rat UTx was used with the uterus donors being of the Dark Agouti strain and the recipient being of the Lewis strain. For mating and to prove fertility, males Sprague-Dawley rats were used. The surgery involved an orthotopic placement of the allograft with, apart from vascular connections end-to-side on the common iliac vessels, also anastomosis between the right uterine horn of the graft and likewise on the vaginal rim. The surgical procedure was essentially the same as used in Paper II.

Results

In a first set of 10 animals, the pelvic and the vascular anatomy of these strains were specifically studied and the surgery was optimised. There was a learning curve with deaths during surgery of animal 4-6 but with the last 4 pre-study animals surviving until 7 days after surgery with adequate gross morphology of the graft. There were two control groups included in the study. These consisted of sham-surgery with no tacrolimus or sham-surgery with tacrolimus. The animals received tacrolimus by a mini-osmotic pump and the tacrolimus blood levels were in the range between around 5 and 25ng/ml.

The pregnancy rates were similarly in the UTx and tacrolimus-treated group and in the sham and tacrolimus-treated group. There was a tendency to somewhat higher pregnancy rate in the sham-group with surgery but not receiving tacrolimus treatment. In the experiments, caesarean section was done at day 17. The number of foetuses were similar in the 3 groups, but with a variation between 0-5. It should be noted that the rate of resorbed pregnancies were higher in pregnant rats of the UTx group receiving tacrolimus compared to the controls.

Comments

This study represents the first study ever that has demonstrated fertility after allogeneic UTx. As stated above, trials of allogeneic UTx with immunosuppression had been performed in the dog (103), rabbit (82) and the rhesus macaque (102). In recent times, monotherapy with tacrolimus after allogeneic UTx has been tested in the baboon (106) and in the rabbit (105). In these latter, studies the drug was administered daily by the oral or intramuscular route. Moreover, daily sc administration of cyclosporine was tested in the allogeneic UTx model in the mouse (83). In the present, study mini-osmotic pumps were used for drug administration. This way of administration to the extracellular space in the subcutis is based on the principle that a constant dose of drug is delivered continuously by osmosis and that this will maintain the target therapeutic level at a fairly constant concentration. This system has been extensively tested in the rat for administration of pharmacological agents such as cyclosporine (107, 108) and for administration of hormones such as estradiol (109, 110)

The reason for terminating the pregnancies at day 17 of pregnancy was both to follow the ethical application protocol, with a step-by-step approach enabling demonstration of pregnancy before evaluating birth and offspring, and with the added benefit of enabling investigation to assess the number of resorbed pregnancies.

The somewhat higher rates of resorbed pregnancies in the uterus transplantation UTxgroup as well as the low pregnancy rate in the groups receiving tacrolimus indicate that this immunosuppressant may in some way negatively affect the pregnancy potential of the rats. In addition, the ischemia reperfusion injury occurring during the time of revascularization of the graft can affect the pregnancy potential. Other factors related to the surgery, as stated in comments to Paper II, can also decrease the fertility rate in these animals.

After presenting this study, demonstrations of pregnancy after allogenic UTx in the sheep was reported by Ramirez and coworkers (111) and thus this initial observation have been extended to other animals. The results of the present study represents the first

demonstration of pregnancy after allogeneic UTx in any species and further studies have to follow to also look at live offspring and development in allogeneic UTx models of different species.

Paper VI

Background

During the last decade, much research efforts have been made in the area of UTx, with specific studies aiming at developing and optimizing procedures such as surgery (59, 100, 112, 113) and ischemic preservation (114, 115). In many of these studies, an important factor to test has been to assess whether the tissue is viable or not. Thus, many studies have relied on histology on the light microscopic level (62, 116), but this is most likely a fairly insensitive test of the normality of the tissue, since early signs of cell death is not detected. In one study on human uterine tissue, electron microscopy was used to assess the viability of the tissue after 24h of cold ischemic preservation in various solutions (71), and this method was able to detect nuclear changes if preservation had occurred in a non-preservation solution. In the same paper, contractility was used to asses if the tissue was functional after a long period of ischemia. However, the function of the uterus is naturally not only to contract but to be able to host a pregnancy and to provide the optimal conditions for implantation and placentation. Thus, the normality of these procedures have been demonstrated after syngeneic UTx in the mouse (60, 117) and the rat (Paper II). Moreover, autotransplantation of the sheep uterus and adnexa (118), with an extended warm ischemic time of 3h, was compatible with normal pregnancies in this animal species with a uterine size of similar size as the human. Recently, one live birth was shown after autologous UTx in a non-human primate species (101).

The situation after allogeneic UTx is naturally more complicated than after syngeneic or aoutologous UTx, since also immunosuppression and rejection mechansims are added factors that may disturb any pregnancy. There are only two studies that so far have demonstrated pregnancy in this allogeneic setting. In the study by Ramirez and coworkers (119), three pregnancies were initially accomplished in their sheep UTx model. The pregnancies were established after embryo transfer. One pregnancy was established in the oviduct and out of natural reasons this pregnancy could not proceed. Another of the three pregnancies ended in miscarriage. The third pregnancy continued well into the third trimester, when a lamb of appropriate size was born. However, life support was only continued for a short term.

In the present study we evaluated the postnatal condition of rats born from allogeneic uterus graft under immunosuppression. Lewis rats served as donors and PVG rate were recipients. The transplantation was by microvascular anastomosis on the common iliacs

and the immunosuppression was by tacrolimus. Two months after transplantation and initiation of immunosuppression by tacrolimus, the transplanted rats were placed for mating and pregnancies were recorded. Two sham-groups were included, one which was subjected to sham-surgery and another which underwent sham surgery under tacrolimus. The pregnancies went to term and the mature fetuses were delivered by caesarean section.

Results

The pregnancy rate was similar in all the three groups with 70% in the sham-surgery group, 80% in the sham group treated with tacrolimus and 50% in the UTx group treated with tacrolimus. It was a fairly large variation in the number of pups in the three different groups, but the birth weight was similar and around 5g in all groups. The female offspring groups were similar during comparison during 100 days. However, the male offspring from the UTx were a little heavier at this stage.

Comments

This is the first study ever that has evaluated the birth weight and the postnatal development of offspring after allogeneic UTx. The offspring has been subjected to many unphysiological factors that may affect their growth in the transplanted uterus but which also may change the growth after birth. The transplanted uterus has been subjected to tissue trauma during the surgical procedure of transplantation and ischemic events may also affect its state after transplantation. After transplantation, the blood flow to the transplanted uterus could be affected due to inability of the anastomosis site to expand in parallel to the hemodynamic changes of pregnancy. Moreover, it is uncertain whether lymphatic drainage and nerve supply of a transplanted uterus are reestablished. All the factors mentioned also exist after syngeneic or autologous UTx and the demonstration of pregnancy in the syngeneic rat UTx model (Paper II) and after autotransplantation in the ewe (77) and the cynomolgus macaque (101) points towards that the UTx methodologies that have been developed are satisfying formal surgical standpoint. In the present study also immunosuppression and possible low grade rejection are added factors that may negatively influence a pregnancy.

The use of tacrolimus to prevent rejection in the allogeneic model had proved to be effective in the experiments of Paper III, although the time period of that paper was only 2 weeks. In the experiments involving allogeneic UTx in the sheep and offspring, cyclosporine monotherapy was used (119).

The results of the present study point towards a normal development of the rats concerning the first 3 months. However, an interesting observation which should be followed up in further studies is that the male rats showed somewhat greater weight gain.

The explanation to this is unclear and naturally a larger cohort of offspring is necessary to establish whether this is a real difference. Further studies should also be made to assess these offspring up into adulthood concerning not only growth but several physiological parameters.

General discussion

The first human transplantation case was performed in Saudi-Arabia in year 2000. This case was published in year 2002 (58) and involved transplantation of a uterus from a 46 years old live donor to a 26 years old patient who had lost her uterus some years earlier at emergency peripartum hysterectomy. The procedure to recover the uterus was similar to a hysterectomy, but involved some dissection of the uterus on the donor. Nevertheless, fairly short lengths of the uterine veins and arteries were attached the uterus that was harvested and back-table preparation had to involve elongation of both the uterine veins and the arteries with saphenous vein grafts. The surgery in the recipient went uneventful but the uterus had to be removed after 3 months because of uterine necrosis.

Many researchers and clinicians throughout the world thought this was a premature attempt to transplant a human uterus since the research group had no previous basic research background concerning UTx in animals and several major issues were not properly examined prior to this extensive surgical procedure in the human. The issues that should be investigated in animal settings before clinical introduction relates to the surgical techniques used at uterus recovery from the donor, the surgery of the recipient, the vascular anastomosis, the tolerability to ischemia and reperfusion, suitable immunosuppression and fertility in a transplanted uterus. Many of these aspects were investigated in the present thesis

The specific results of each paper are commented in the Results and Comments section of this thesis. In this section I have chosen to discuss some general issues, of relevance to the results that were obtained and to future directions of research concerning UTx in the rat model.

The rat as an experimental animal

The research group that I belong to has developed several animal models in a research-based approach towards human uterus transplantation. During recent years the rat has proved to be the most suitable small animal model and we have complimented studies in the rat with those in non-human primate species (120, 121). Other research groups in the world have mainly used the sheep (122), the pig (6, 61) and the rabbit (105). The focus of this thesis was to develop the rat model for uterus transplantation research.

There are many possible advantages with the rat model. The laboratory rat is one of the most commonly used experimental animals. It serves as a model for the analysis of a number of important biomedical issues such as metabolism, organ transplantation and

immunology. Since the knowledge about the rat is vast it has a unique advantage as model system for human diseases and also human surgical interventions.

The size of the rat makes it ideal for physiological manipulations and also for surgery, in comparison to the much smaller laboratory mouse.

Most laboratory rats belong to the Norway rat (*Rattus Norvegicus*). The Norway rat was one of the first mammalian species to be domesticated for scientific purposes. Today it is the second most widely used experimental animal in biomedical research, with only the mouse being used more frequently. An advantage of the rat is the existence of genetically defined strains. Concerning transplantation research, both research on various organ transplantation and immunology has established the rat as one of the favourite research tools. The major histocompatibility complex (MHC) has been investigated in the rat and it is now called RT1. This RT1 system is located on chromosome 17 (123) and has recently been further characterized (124). Of importance is of course the T-cell composition of the rat immune system, since T-cells are instrumental in rejection. There is presence of both CD4 positive and CD8 positive cells and importantly IL-2 is involved in the T-cell development also in rats (125). This is of importance since one effect of calcineurin inhibitors is to suppress IL-2 synthesis. There exist specific strains of rats that spontaneously acquire diseases such as hypertension, stroke, insulin dependent diabetes, spontaneous tumors and arthritis.

It has been more difficult to develop efficient transgenic technology in the rat as compared to the mouse. This technical problem has recently been overcome and knockout technology and transgenic overexpression of rats is now also possible. Some good features of rat biology in comparison to mouse models are more human like physiological responses to some diseases such as arthritis, cancer and hypertension. Moreover, the larger size makes it more suitable for surgical manipulation and repeated blood sampling. These latter things will of course have relevance to transplantation research.

The first method for transplantation in the rat was cardiac transplants in 1964 (126, 127). In this initial model, the heart was heterotopically transplanted by connecting the recipient's abdominal aorta and the donors descending aorta using end-to-end technique. Later on, this transplantation method was modified to use the abdominal vessels (128). During recent years, the cardiac transplantation model that has been most widely used is a technique for auxillary transplantation in the rat, where the native heart is kept in place (129). In the same paper, an auxillary heart and lung transplantation was published. Just one year later, an orthotopic non-auxillary heart and lung transplantation was published, but more than 300 transplant trials were done before any survival of the rat was accomplished (130). The technique has then been modified and the survival rate is now over 80% after orthotopic lung and heart transplantation in the syngeneic rat. The UTx

models, as developed in the present thesis (Paper I, II), has naturally benefitted in their development from these early pioneering studies in experimental transplantation.

During recent years rat models for liver transplantation (131) and intestinal transplantation (132) have been developed and successfully been used to study several aspects of the transplantation procedure.

Syngeneic UTx and fertility

Experimental syngeneic UTx models can be used to separate the effects of surgery, ischemia and the new anatomical placement of a transplanted uterus from effects of rejection and immunosuppressive agents, which add to the complexity of allogeneic transplantation. The donor and recipient in syngeneic transplantation will be virtually genetically identical, since they would be of the same inbred strain of an experimental animal, or in the human be identical twins.

Most inbred animal strains exist in the rodent animal species. The mouse has the advantage over the rat, since it is easy to gene-modify mice and there is a great abundance of recombinant proteins and monoclonal antibodies in this species. The weight of the adult mouse (20-25g) is conversely a disadvantage in surgical procedures such as transplantation, in comparison to the adult rat (Paper I-VI) with a weight at least 4 times that of the mouse.

The first rodent model, which was thoroughly explored concerning UTx and fertility, was the mouse model. In the primary mouse UTx study, F1 hybrids of females of the inbred strains C57BL/6 and CBA/ca were used (59) and the surgery included a model of heterotopic UTx, where the native uterus was kept in situ as an internal control. The transplantation was performed by an end-to-side anastomosis of the vena cava and aorta of the graft and the recipient. Great microsurgical skills were needed since the lumina of the aorta and vena cava were only about 0.7 mm and 1.5 mm, respectively. These microsurgical skills could not be fully replicated by others and that is one reason why rat models of UTx were developed (Paper I, II). In the initial model the uterine cervix of the graft was positioned intraabdominally to avoid possible infections through contact with the exterior. In this initial mouse study (59), the fertility potential was tested only in one of the experimental animals. At about 2 weeks after UTx, blastocysts were retrieved from a naturally mated mouse. The blastocysts were transferred to the uterine cavity of both the transplanted graft and the native uterus, after preparing the transplanted mouse for a pseudopregnancy, with mating it with a sterile male mouse. The embryo transfer (both to native and grafted uterus) was performed via a midline laparotomy and then transmyomterially through a thin cannula. Three blastocysts were placed inside each uterus cavity and the animal was euthanized, 10 days after embryo transfer. One foetus

was seen in the graft, which also had an absorbed pregnancy. The paper (59)], represents the initial t proof-of-principle study in relation to the fertility and UTx.

In a follow-up paper, the UTx procedure was modified so that the cervix graft was connected to the skin of the mouse to create a cervical-cutaneous stoma (60), in order to avoid uterine swelling, secondary to accumulated intraluminal mucus, which was seen in the initial mouse study. This model is similar to the rat UTx model of Paper I, where also the vascular anastomosis sites were the infrarenal abdominal aorta and caval vein.

The pregnancy rate of transplanted animals, carrying both native and grafted uteri, were compared with sham-surgery animals. The mice received embryos during the interval from 1-3 weeks after transplantation, with blastocysts (3-6 to each uterus horn) inserted transmyometrially (133). The first set of animals was evaluated concerning fertility at 2-3 days before anticipated parturition. The pregnancy rate was similar in the native (75%) and the grafted (66%) uterus of the transplanted animals as compared to control animals (66%). This can be compared to a pregnancy rate of 47% in the syngeneic rat UTx group of Paper II. It should be noted that Paper II involved spontaneous mating and this mouse study (60), involved pregnancies after embryo transfer. Importantly, the median numbers of foetuses in these pregnant mice (60) pregnant animals were similar in the groups with 4, 4 and 3 in native, grafted and control uterus, respectively. The mouse study also examined the weight of the foetuses at day 18 and that of the placenta, with similar weights recorded in all groups. In the second series of experiment in the mouse UTx paper (60), pregnancies went to full term and pups were delivered spontaneously, but caesarean section delivery was attempted from the grafted uterus. In some cases delayed spontaneous deliveries occurred from the graft. An explanation for the delay may be mechanical factors, with slow cervical dilation at the point of passage through the skin but poor nerve supply of the transplanted uterus and the heterotopic position of the organ may also influence the results. In Paper II, the rate of successful deliveries was markedly lower than in the control group and denervation of the uterus may also be one factor that influence the results in that orthotopic transplantation method.

The postnatal and later growth of the mouse offspring was followed for 8 postnatal weeks. The growth was normal, as compared to animals from native uteri and from control animals. Concerning the results of Paper II, growth trajectory of the live births from the sham-surgery group and the UTx group was similar, though any firm conclusions should not be made since the number of live births in the UTx group was very low. In order to rule out that the native uterus of the mouse would influence the pregnancy potential of the grafted uterus, some animals underwent UTx with the native uterus removed (60). This is a situation as in Paper II, but with the difference of placement of the grafted uterus at heterotopic and orthotopic positions, respectively. Pregnancies were seen in the mice that had no native uterus, showing that pregnancy

potential of the graft was independent on input from the native uterus. The fertility of offspring of both genders from the grafted uterus was normal and the second generation offspring from these mice showed normal birthweight (60). In addaition, one study in the syngeneic mouse model has demnostrated normal fertility also after preservation of the uterus in a cold environment, during the time from removal to transplantation (117)

In Paper II, pregnancy potential of the syngeneic uterine graft, with one uterine horn excised, was compared to sham-operated rats that only underwent unilateral uterine horn excision. In the series of 19 animals in each group, pregnancy rate was similar with 11 rats in the UTx group versus 12 in the sham-surgery group. The median numbers of pups were 3 in the UTx group and 3.5 in the sham group. However, as mentioned above the UTx group had lower rate of successful deliveries. An explanation to this may be a protracted labour in the transplanted uterus, caused by denervation. This assumption was based on a previous paper showing that the sensory innervation of the uterus is necessary for a normal delivery in the rat (134).

The results of these syngeneic rodent UTx models, as discussed above, are important as the first demonstrations of the principle of fertility potential in a transplanted uterus. However, being rodent models, the experimental situation is certainly different from a human situation concerning surgery and also pregnancy length as well as placentation. Moreover, the extra strain of being an allogeneic graft has not been evaluated. This is further discussed below.

Allogeneic UTx and fertility

The allogeneic UTx situation is obviously more multifaceted than syngeneic and UTx, since the effects of rejection and immunosuppression also are factors that may affect the fertility potential of a transplanted uterus.

The results of Paper V, was the first description of pregnancy after allogeneic UTx, which of course is an important proof-of principle. The surgical technique used in this study was modified from the syngeneic rat model (Paper II). The Dark Agouti (DA, RT-1^a) uterus donors were operated by a midline laparotomy to surgically isolate the uterus, with the right uterine horn excised. The Lewis ($RT1^l$) uterus recipienst were prepared for UTx by hysterectomy and dissections of the common iliac artery and vein on the right side were performed, to be used as vascular anastomosis sites. The immunosuppressant given to prevent uterine graft rejection was tacrolimus at a dose of 0.5 mg·kg-1·day-1. In addition to the transplanted group (UTx-Tac), two control groups were included in this study: 1) rats undergoing just sham surgery (hemihysterectomy; Sham) and 2) rats undergoing sham surgery and the same immunosuppressive protocol as the transplanted group

(Sham-Tac). In this Paper V, spontaneous mating occurred after introduction of experimental animals to males of proven-fertility. The number of foetuses and implantation sites were counted at the time of cesarean sections on pregnancy day 17. Pregnancy rates were slightly (but insignificantly, possibly due to the small sample size (n=19)) decreased in the groups undergoing immunosuppression: 62.5% in the UTx and tacrolimus treated group, 60% in the sham-group with tacrolimus treatment, as compared to 83% in the sham-surgery group. This study was only designed to prove that pregnancy was possible after allogenic UTx and to evaluate the possible impact on implantation rate. This is a step-by-step method we have used in studies on other aspects of experimental UTx.

In the follow-up paper (Paper VI) Lewis ($RT1^l$) rats were used as donors and PVG ($RT1^c$) rats as recipients. The pregnancy rates did not show any significant differences between the groups with 50% in the UTx and tacrolimus treated group, 80% in the sham-group with tacrolimus treatment and 70% in the sham-surgery group, but the birth weights of the pups were similar in the groups. As shown in Paper VI, the growth of offspring from the experimental groups was similar in the control groups during the first months after transplantation. However, a small elevation in weight was seen in the male offspring of the UTx group with tacrolimus treatment at postnatal day 100. This should be explored further whether any progressive weight deviation occurs.

There also exists some experience of allogeneic UTx in a larger animal. In 2011, another group reported two pregnancies and one birth after allogeneic UTx in the sheep (119), using a previously published surgical model (62). Via a small subumbilical incision, the uterus with short uterine vessel pedicles was retrieved from one animal and placed in another animal that underwent the same surgical treatment (62). The anastomoses technique used was end-to-end on the uterine arteries and veins and the vaginal vault of the graft was also end-to-end anastomosed to the vaginal rim of the recipient. The experiment involved allogeneic transplantations between Sudanese and Ethiopian breeds (119). However, it should be noted that sheep bred in farms may diminish the functional allogenicity of the transplantation (135) and it was not full reported concerning their allogeneicity. The recipients were treated orally with cyclosporine A and intramuscularly with prednisone, starting two days in advance of UTx. In the study prednisone treatment was discontinued after 2 weeks post-transplantation and satisfactory levels of cyclosporine were reached by day 5. Twelve animals underwent UTx with 8 surviving the procedure. Embryo-transfer was done in 5 animals with normal cyclicity and a normal graft at second-look surgery (119). Three pregnancies were reported. One pregnancy was an ectopic pregnancy requiring removal of the uterus, another resulted in fetal demise at

105 days of gestation and the last pregnancy was in utero and developed normally. This resulted in delivery of a fully developed lamb at 138 days, via cesarean section.

Future directions of UTx research in the rat model

The results of this thesis have demonstrated that the rat UTx model is a reproducible model, which can be mastered by previously untrained microsurgeons and with fertility after spontaneous mating. During recent years also other animal models have been developed and importantly are the non-human primate models. However, these non-human primate models are very costly and experiments may be difficult to design due to that there is a fairly large inter-individual variation in outbred settings. Thus, the rat and the mouse model may have their places in further UTx research.

The importance of these rodent models of UTx will lay in that a larger number of animals can be used in an experimental design. Moreover, the restricted pregnancy time and the restricted time between generations make it suitable for research on reproductive factors, such as fertility after Utx. The studies in the thesis have shown that the surgery can be mastered well, both in the heterotopic (Paper I) and orthotopic (Paper II) UTx-model. However, the surgery could possibly be developed somewhat by further training and modification of the procedure, to increase the graft survival. Especailly in the allogeneic transplantations, initial graft survival has been modest (Paper IV).

The most important aspect now in the rat UTx research should be to assess whether the offspring from uterus transplanted animals are fully normal. In Paper V, fertility was demonstrated after allogeneic uterus transplantation. In the follow-up paper (Paper VI) it was demonstrated that the birth weight was normal in the offspring from the transplanted group. However at 100 days of age, the offspring male rats from the transplanted group proved to be somewhat larger than the offspring from the control group (Paper VI). Naturally, weight is just one very crude variable of the rats and several aspects should be studied on these offsprings.

In particularly, the placenta should be studied with histology and immunohistochemistry towards important placental proteins such as for example steroid receptors, steroidogenic enzymes and vascular markers. It is known that birth weight influences adulthood conditions and diseases such as the metabolic syndrome and cardiovascular diseases. The metabolism of the offspring could be studied at different ages of the animals. This could be done both by investigating the basal metabolism in metabolic cages. Moreover, glucose tolerance could be studied by clamp techniques to ascertain that these rats are not pre-diabetic. Another important factor is the body composition. A way to study this is by

dual energy X-ray absorbtion (DEXA) which can determine both lean body mass as well as fat mass. The basal metabolic status can also be evaluated by indirect calorimetry and oxygen consumption rate, usually performed in metabolic cages over 24 hours.

Concerning cardiovascular monitoring, blood pressure should be measured at different ages and functional electrocardiography can also be done. It is known that calcineurin inhbibitors affects the kidney and the kidney function should also be evaluated. This can easily be accomplished in metabolic cages, where urine sampling can be arranged. The urine should be analysed in detail to describe ionic and possible altered protein composition, as well as creatinine levels. Since the allogeneic offspring have been subjected to immunomodulator drugs during their foetal development, thorough immunological tests should be done concerning both numbers and function of all different immune cell components.

It could not be excluded that the development in an allogeneic uterus graft can affect the behaviour. There exist tests for both locomotor activity and anxiety in the rats and that could be used in these follow-up studies. It should also be ascertained that females and males have full fertility after allogeneic uterus transplant. This test should include normal mating experiments with males and females of proven fertility.

When the animals are euthanized, several tissues could be collected for detailed histological analysis and immunohistochemistry. Important is to rule any increased rate of tumour development in tissues examined, but also to observe if any other structural changes are present. The especially important tissues would be the kidney, liver, heart and of course the reproductive tract. Aorta may also be of importance to examine to rule out the presence of aortic sclerosis.

Concluding remarks

These studies on UTx in the rat model represent the first detailed descriptive studies on UTx in this species and they have also advanced the UTx field further, especially concerning studies of allogeneic UTx. They should serve as the fundament for further studies, not just exploring the offspring, but perhaps also to further optimized immunosuppression and preservation conditions at transplantation.

Acknowledgements

This thesis work was carried out at the Institute for Clinical science and the Department of Obstetrics and gynecology, Sahlgrenska Academy, University of Gothenburg, during the period of 2009-2012. I want to express my sincere gratitude to a great number of persons for all the support they have given during this period.

My deepest gratitude belongs to Prof. Mats Brännström, my supervisor, who has guided me during my struggle with this thesis. This thesis would never have been possible without his knowledge, ideas and support.

Dr.Caiza Wranning has been a great co-supervisor and friend during all the years of my research. I am particularly grateful for her help during the early days of my work, for teaching me from the basics of how to do everything in the lab. I am grateful to my other co-supervisor **Dr. Randa R. El-Akouri** for all her help, support and for always being there during the last year of my PhD.

Very special thanks to **Johan Mölne** for trying to make me understand the complicated subject inflammation.

Professor **Suchitra Holgersson, Jan Holgersson** and **Michael Olausson** with their staff have furnished an excellent research environment. A special thanks to all of them for providing an inspiring research environment.

Special thanks go also to **Cesar Diaz-Garcia** for the support and companionship during the time working at EBM, during my half time seminar and for the significant help with medical statistics during my introductory course.

I am very much grateful to **Ann Wallin,** who always found the time and patience to help me with several problems I faced in my research and give her best suggestions.

Thanks to **Mats Hellström** who as a good friend, was always willing to help and give his best suggestions.

Thanks to **Liza johannssonn, Klaus Grouth** and all other members in the uterus transplantation research group.

All of my colleagues and friends at transplantation centrum, for always providing a helping hand when I needed. Special thanks to **Margareta Filipsson**, **Nidia Hernandez**, **Pradeep Patil**, **Ketky Methe**, **Nikhil Nayakawde**, **Premaratne Goditha, Simona Oltean** and **Ann Novotny**

Special thanks to **Mehai Oltean** for scientific advice.

Thanks **Mariette** for helping me with animal care. Very special thanks to **Abdulhussain Haamid** for always willing to help.

Thanks **Ingrid Prim-Rizopulos** for doing the computer work with this thesis.

My parents, **Shainuddin Fakir and Lutfun Nahar**, for teaching me to never give up. All the support they have provided me over the years is the greatest gift anyone has ever given me.

Sohana Irin Beethi, my sister, **Nazibul Haque Toslim**, my younger brother and my older brother **Lutful Haque Tofayal** who always encourage me with their best wishes to achieve at the highest levels. Thank you for your endless love, support and for always standing by me.

I owe my deepest gratitude to my grandfather **Abdul Latif**, grandmother **Anuara Begum**, aunt **Mariom** and **Norunnahar Hena** and ancle **Anuarul Islam** for their endless love, support and confidence in me.

Lastly**, Mohammad Aurangojeb** my beloved husband. I cannot find words to thank you enough for supporting me and being in my life. With you by my side I always feel stronger. Your contribution to this thesis is immense; I could never have done this without you.

References

1. Schneeberger S, Landin L, Jableki J, Butler P, Hoehnke C, Brandacher G *et al.* Achievements and challenges in composite tissue allotransplantation. Transpl Int 2011;24:760-9.

2. Aarts JWM, van Empel IWH, Boivin J, Nelen WL, Kremer JAM, Verhaak CM. Relationship between quality of life and distress in infertility: a validation study of the Dutch FertiQoL. Hum Reprod 2011;26:1112-8.

3. Forti G, Krausz C. Clinical review 100: Evaluation and treatment of the infertile couple. J Clin Endocrinol Metab 1998;83:4177-88.

4. Kilchevsky A, Honig S. Male factor infertility in 2011: Semen quality, sperm selection and hematospermia. Nat Rev Urol 2012;9:68-70.

5. Organization WH. Infertility: a tabulation of available data on prevalence of primary and secondary infertility. Geneva, WHO, Programme on Maternal and Child Health and Family Planning, Division of Family Health 1991.

6. Sieunarine K, Zakaria FB, Boyle DC, Corless DJ, Noakes DE, Lindsay I *et al.* Possibilities for fertility restoration: a new surgical technique. Int Surg 2005;90:249-56.

7. Goldfarb JM, Austin C, Peskin B, Lisbona H, Desai N, de Mola JR. Fifteen years experience with an in-vitro fertilization surrogate gestational pregnancy programme. Hum Reprod 2000;15:1075-8.

8. Quinn MA, Benedet JL, Odicino F, Maisonneuve P, Beller U, Creasman WT *et al.* Carcinoma of the cervix uteri. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. Int J Gynaecol Obstet 2006;95 Suppl 1:S43 -103.

9. Sonoda Y, Abu-Rustum NR, Gemignani ML, Chi DS, Brown CL, Poynor EA *et al.* A fertility-sparing alternative to radical hysterectomy: how many patients may be eligible? Gynecol oncol 2004;95:534-8.

10. Kwee A, Bots ML, Visser GHA, Bruinse HW. Emergency peripartum hysterectomy: A prospective study in The Netherlands. Eur J Obstet Gynecol Reprod Biol 2006;124:187-92.

11. Marino JL, Eskenazi B, Warner M, Samuels S, Vercellini P, Gavoni N *et al.* Uterine leiomyoma and menstrual cycle characteristics in a population-based cohort study. Hum Reprod 2004;19:2350-5.

12. Stewart EA. Uterine fibroids. The Lancet 2001;357:293-8.

13. Borgfeldt C, Andolf E. Transvaginal ultrasonographic findings in the uterus and the endometrium: Low prevalence of leiomyoma in a random sample of women age 25-40 years. Acta Obstet Gynecol Scand 2000;79:202-.

14. Marshall LM, Spiegelman D, Barbieri RL, Goldman MB, Manson JE, Colditz GA *et al.* Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. Obstet Gynecol 1997;90:967-73.

15. Chen CR, Buck GM, Courey NG, Perez KM, Wactawski-Wende J. Risk factors for uterine fibroids among women undergoing tubal sterilization. Am J Epidemiol 2001;153:20-6.

16. Farhi J, Ashkenazi J, Feldberg D, Dicker D, Orvieto R, Ben Rafael Z. Effect of uterine leiomyomata on the results of in-vitro fertilization treatment. Hum Reprod 1995;10:2576-8.

17. Oliveira FG, Abdelmassih R, Abdelmassih VG, Diamond MP, Dozortsev D, Melo NR. Impact of subserosal and intramural uterine fibroids that do not distort the endometrial cavity on the outcome of in vitro fertilization–intracytoplasmic sperm injection. Fertil Steril 2004;81:582-7.

18. Benecke C, Kruger TF, Siebert TI, Van der Merwe JP, Steyn DW. Effect of fibroids on fertility in patients undergoing assisted reproduction. A structured literature review. Gynecol Obstet Invest 2005;59:225-9.

19. Somigliana E, Vercellini P, Daguati R, Pasin R, De Giorgi O, Crosignani PG. Fibroids and female reproduction: a critical analysis of the evidence. Hum Reprod Update 2007;13:465-76.

20. Fernandez H, Sefrioui O, Virelizier C, Gervaise A, Gomel V, Frydman R. Hysteroscopic resection of submucosal myomas in patients with infertility. Hum Reprod 2001;16:1489-92.

21. Al-Inany H. Intrauterine adhesions. An update. Acta Obstet Gynecol Scand 2001;80:986-.

22. Bukulmez O, Yarali H, Gurgan T. Total corporal synechiae due to tuberculosis carry a very poor prognosis following hysteroscopic synechialysis. Hum Reprod 1999;14:1960-1.

23. Friedler S, Margalioth EJ, Kafka I, Yaffe H. Incidence of post-abortion intra-uterine adhesions evaluated by hysteroscopy--a prospective study. Hum Reprod 1993;8:442.

24. Schenker J, Margalioth E. Intrauterine adhesions: an updated appraisal. Fertil Steril 1982;37:593.

25. Fernandez H, Al-Najjar F, Chauveaud-Lambling A, Frydman R, Gervaise A. Fertility after treatment of Asherman's syndrome stage 3 and 4. J Minim Invasive Gynecol 2006;13:398-402.

26. Grimbizis GF, Camus M, Tarlatzis BC, Bontis JN, Devroey P. Clinical implications of uterine malformations and hysteroscopic treatment results. Hum Reprod Update 2001;7:161-74.

27. Raga F, Bauset C, Remohi J, Bonilla-Musoles F, Simón C, Pellicer A. Reproductive impact of congenital Müllerian anomalies. Hum Reprod 1997;12:2277-81.

28. Acién P. Embryological observations on the female genital tract. Hum Reprod 1992;7:437.

29. Byrne J, Nussbaum-Blask A, Taylor WS, Rubin A, Hill M, O'Donnell R *et al.* Prevalence of müllerian duct anomalies detected at ultrasound. Am J Med Genet A 2000;94:9-12.

30. Troiano RN, McCarthy SM. Mullerian duct anomalies: imaging and clinical issues. Radiology 2004;233:19.

31. Saravelos SH, Cocksedge KA, Li T-C. Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal. Hum Reprod Update 2008;14:415-29.

32. Homer HA, Li T-C, Cooke ID. The septate uterus: a review of management and reproductive outcome. Fertil Steril 2000;73:1-14.

33. Pabuçcu R, Gomel V. Reproductive outcome after hysteroscopic metroplasty in women with septate uterus and otherwise unexplained infertility. Fertil Steril 2004;81:1675-8.

34. Buttram VC, Jr. Mullerian anomalies and their management. Fertil Steril 1983;40:159-63.

35. Papp Z, Mezei G, Gavai M, Hupuczi P, Urbancsek J. Reproductive performance after transabdominal metroplasty: a review of 157 consecutive cases. J Reprod Med 2006;51:544-52.

36. Nahum GG. Uterine anomalies. How common are they, and what is their distribution among subtypes? J Reprod Med 1998;43:877-87.

37. Michalas SP. Outcome of pregnancy in women with uterine malformation: evaluation of 62 cases. Int J Gynaecol Obstet 1991;35:215-9.

38. Fedele L, Zamberletti D, Vercellini P, Dorta M, Candiani GB. Reproductive performance of women with unicornuate uterus. Fertil Steril 1987;47:416-9.

39. Donnez J, Nisolle M. Endoscopic laser treatment of uterine malformations. Hum Reprod 1997;12:1381-7.

40. Taylor E, Gomel V. The uterus and fertility. Fertil Steril 2008;89:1-16.

41. Nohara M, Nakayama M, Masamoto H, Nakazato K, Sakumoto K, Kanazawa K. Twin pregnancy in each half of a uterus didelphys with a delivery interval of 66 days. BJOG 2003;110:331-2.

42. Folch M, Pigem I, Konje JC. Mullerian agenesis: etiology, diagnosis, and management. Obstet Gynecol Surv 2000;55:644-9.

43. Oppelt P, Beckmann MW, Renner SP, Kellermann A, Brucker S, Hauser GA *et al.* Clinical aspects of Mayer-Rokitansky-Kuester-Hauser syndrome: recommendations for clinical diagnosis and staging. Hum Reprod 2006;21:792-7.

44. Petrozza JC, Gray MR, Davis AJ, Reindollar RH. Congenital absence of the uterus and vagina is not commonly transmitted as a dominant genetic trait: Outcomes of surrogate pregnancies. Fertil Steril 1997;67:387-9.

45. Game DS, Warrens AN, Lechler RI. Rejection mechanisms in transplantation. Wien Klin Wochenschr 2001;113:832-8.

46. Busch GJ, Martins AC, Hollenberg NK, Wilson RE, Colman RW. A primate model of hyperacute renal allograft rejection. Am J Pathol 1975;79:31-56.

47. Forbes RD, Kuramochi T, Guttmann RD, Klassen J, Knaack J. A controlled sequential morphologic study of hyperacute cardiac allograft rejection in the rat. Lab Invest 1975;33:280-8.

48. Morris P, Bradley A, Doyal L, Earley M, Hagen P, Milling M *et al.* Face transplantation: a review of the technical, immunological, psychological and clinical issues with recommendations for good practice. Transplantation 2007;83:109-28.

49. Murase N, Demetris AJ, Woo J, Tanabe M, Furuya T, Todo S *et al.* Graft-versus-host disease after brown Norway-to-Lewis and Lewis-to-Brown Norway rat intestinal transplantation under FK506. Transplantation 1993;55:1-7.

50. Easterling KJ, Trumble TE. The treatment of peripheral nerve injuries using irradiated allografts and temporary host immunosuppression (in a rat model). J Reconstr Microsurg 1990;6:301-7; discussion 9-10.

51. Mizuta T, Fujii Y, Minami M, Tanaka S, Utsumi T, Kosaka H *et al.* Increased nitric oxide levels in exhaled air of rat lung allografts. J Thorac Cardiovasc Surg 1997;113:830-5.

52. Murase N, Demetris AJ, Woo J, Furuya T, Nalesnik M, Tanabe M *et al.* Lymphocyte traffic and graft-versus-host disease after fully allogeneic small bowel transplantation. Transplant Proc 1991;23:3246-7.

53. Schmidt A, Sucke J, Fuchs-Moll G, Freitag P, Hirschburger M, Kaufmann A *et al.* Macrophages in experimental rat lung isografts and allografts: infiltration and proliferation in situ. J Leukoc Biol 2007;81:186-94.

54. Fujishiro J, Suzuki C, Kudou S, Yasue T, Hakamata Y, Takahashi M *et al.* Change from cyclosporine to combination therapy of mycophenolic acid with the new sphingosine-1-phosphate receptor agonist, KRP-203, prevents host nephrotoxicity and transplant vasculopathy in rats. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation 2006;25:825-33.

55. Tanabe M, Murase N, Demetris AJ, Hoffman RA, Nakamura K, Fujisaki S *et al.* The influence of donor and recipient strains in isolated small bowel transplantation in rats. Transplant Proc 1994;26:3733-40.

56. Brown CB, Luciano AA, Martin D, Peers E, Scrimgeour A, diZerega GS. Adept (icodextrin 4% solution) reduces adhesions after laparoscopic surgery for adhesiolysis: a double-blind, randomized, controlled study. Fertil Steril 2007;88:1413-26.

57. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

58. Fageeh W, Raffa H, Jabbad H, Marzouki A. Transplantation of the human uterus. Int J Gynaecol Obstet 2002;76:245-51.

59. Racho El-Akouri R, Kurlberg G, Dindelegan G, Molne J, Wallin A, Brannstrom M. Heterotopic uterine transplantation by vascular anastomosis in the mouse. J Endocrinol 2002;174:157-66.

60. Racho El-Akouri R, Kurlberg G, Brannstrom M. Successful uterine transplantation in the mouse: pregnancy and post-natal development of offspring. Hum Reprod 2003;18:2018-23.

61. Avison DL, DeFaria W, Tryphonopoulos P, Tekin A, Attia GR, Takahashi H *et al.* Heterotopic uterus transplantation in a swine model. Transplantation 2009;88:465-9.

62. Ramirez ER, Ramirez DK, Pillari VT, Vasquez H, Ramirez HA. Modified uterine transplant procedure in the sheep model. J Minim Invasive Gynecol 2008;15:311-4.

63. Martins PN. Learning curve, surgical results and operative complications for kidney transplantation in mice. Microsurgery 2006;26:590-3.

64. Kawahara K, Sutherland DE, Rynasiewicz JJ, Najarian JS. Prolongation of heterotopic cardiac allografts in rats by cyclosporin A. Surgery 1980;88:594-600.

65. Giardina JJ, Malinowska K, Pifarre R, Hwang MH, Robinson JA, Loeb HS *et al.* Use of cyclosporine in the mouse heterotopic heart transplant model. J Heart Transplant 1990;9:106-13.

66. Wranning CA, El-Akouri RR, Groth K, Molne J, Parra AK, Brannstrom M. Rejection of the transplanted uterus is suppressed by cyclosporine A in a semi-allogeneic mouse model. Hum Reprod 2006;22:372-9.

67. Zhong R. Organ transplantation in mice: current status and future prospects. Microsurgery 1999;19:52-5.

68. Gillett WR. Artefactual loss of human ovarian surface epithelium: potential clinical significance. Reprod Fertil Dev 1991;3:93-8.

69. Welbourn CR, Goldman G, Paterson IS, Valeri CR, Shepro D, Hechtman HB. Pathophysiology of ischaemia reperfusion injury: central role of the neutrophil. Br J Surg 1991;78:651-5.

70. Buonocore S, Surquin M, Le Moine A, Abramowicz D, Flamand V, Goldman M. Amplification of T-cell responses by neutrophils: relevance to allograft immunity. Immunol Lett 2004;94:163-6.

71. Wranning CA, Molne J, El-Akouri RR, Kurlberg G, Brannstrom M. Short-term ischaemic storage of human uterine myometrium--basic studies towards uterine transplantation. Hum Reprod 2005;20:2736-44.

72. Diaz-Garcia C, Akhi SN, Martínez-Varea A, Brännström M. The effect of warm ischemia at uterus transplantation in a rat model. Acta Obstet Gynecol Scand 2012Epub.

73. Jungebluth P, Gudbjartsson T, Le Guyader S, Henriksson G, Hermanson O, Juto JE *et al.* Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. Lancet 2011;378:1997-2004.

74. Olausson M, Sumitran-Holgersson S, Patil PB, Kuna VK, Chougule P, Hernandez NM *et al.* Transplantation of an allogeneic vein bioengineered with autologous stem cells: a proof-of-concept study. Lancet 2012; Epub.

75. Bulletti C, Palagiano A, Pace C, Cerni A, Borini A, de Ziegler D. The artificial womb. Ann N Y Acad Sci 2011;1221:124-8.

76. Engineering endometrial tissue.

http://www.obgyn.net/displaytranscript.asp?page=/avtranscripts/asrm2001-liu. October 22-24, 2001.

77. Wranning C, Marcickiewicz J, Enskog A, Dahm-Kähler P, Hanafy A, Brännström M. Fertility after autologous ovine uterine-tubal-ovarian transplantation by vascular anastomosis to the external iliac vessels. Hum Reprod. 2010 Aug; 25(8):1973-9

78. Lerut J, Sanchez-Fueyo A. An Appraisal of Tolerance in Liver Transplantation. Am J Transplant 2006;6:1774.

79. Takatsuki M, Uemoto S, Inomata Y, Egawa H, Kiuchi T, Fujita S *et al.* Weaning of immunosuppression in living donor liver transplant recipients. Transplantation 2001;72:449.

80. Lee JH, Lee S-K, Lee HJ, Seo JM, Joh JW, Kim SJ *et al.* Withdrawal of Immunosuppression in Pediatric Liver Transplant Recipients in Korea. Yonsei Med J 2009;50:784-8.

81. Scott JR, Anderson WR, Kling TG, Yannone ME. Uterine Transplantation in Dogs. Gynecol Obstet Invest 1970;1:140-8.

82. Confino E, Vermesh M, Thomas W, Gleicher N. Non-vascular transplantation of the rabbit uterus. Int J Gynaecol Obstet 1986;24:321-5.

83. Wranning CA, El-Akouri RR, Groth K, Molne J, Parra AK, Brannstrom M. Rejection of the transplanted uterus is suppressed by cyclosporine A in a semi-allogeneic mouse model. Hum Reprod 2007;22:372-9.

84. Groth K, Akhi SN, Molne J, Wranning CA, Brannstrom M. Effects of immunosuppression by cyclosporine A on allogenic uterine transplant in the rat. Eur J Obstet Gynecol Reprod Biol 2012;163:97-103.

85. Landin L, Cavadas PC, Gonzalez E, Rodriguez JC, Caballero A. Functional outcome after facial allograft transplantation in rats. J Plast Reconstr Aesthet Surg 2008;61:1034-43.

86. Siemionow M, Ozer K. Advances in composite tissue allograft transplantation as related to the hand and upper extremity. J Hand Surg Am. 2002;27:565-80.

87. Muramatsu K, Doi K, Kawai S. Limb allotransplantation in rats: combined immunosuppression by FK-506 and 15-deoxyspergualin. J Hand Surg Am 1999;24:586-93.

88. Angiolillo AL, Sgadari C, Taub DD, Liao F, Farber JM, Maheshwari S *et al.* Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. J Exp Med 1995;182:155-62.

89. Sgadari C, Angiolillo AL, Tosato G. Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10. Blood 1996 May 1;87(9):3877-82.

90. Imakawa K, Nagaoka K, Nojima H, Hara Y, Christenson RK. Changes in immune cell distribution and IL-10 production are regulated through endometrial IP-10 expression in the goat uterus. Am J Reprod Immunol 2005;53:54-64.

91. van Mourik MSM, Macklon NS, Heijnen CJ. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol 2009;85:4-19.

92. Gnainsky Y, Granot I, Aldo PB, Barash A, Or Y, Schechtman E *et al.* Local injury of the endometrium induces an inflammatory response that promotes successful implantation. Fertil Steril 2010;94:2030-6.

93. Johannesson L, Enskog A, Molne J, Diaz-Garcia C, Hanafy A, Dahm-Kahler P *et al.* Preclinical report on allogeneic uterus transplantation in non-human primates. Hum Reprod 2012. Epub

94. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012;93:1-10.

95. Mohammad Reza G, Abdolreza H. Chronic Allograft Dysfunction Major Contributing Factors. Iran J Kidney Dis 2012;6:88-93.

96. Uslu A, Nart A. Treatment of First Acute Rejection Episode: Systematic Review of Level I Evidence. Transplant Proc 2011;43:841-6.

97. El-Akouri RR, Molne J, Groth K, Kurlberg G, Brannstrom M. Rejection patterns in allogeneic uterus transplantation in the mouse. Hum Reprod 2006;21:436-42.

98. Groth K, Akouri R, Wranning CA, Molne J, Brannstrom M. Rejection of allogenic uterus transplant in the mouse: time-dependent and site-specific infiltration of leukocyte subtypes. Hum Reprod 2009;24:2746-54.

99. Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 2008;36:375-84.

100. Enskog A, Johannesson L, Chai DC, Dahm-Kahler P, Marcickiewicz J, Nyachieo A *et al.* Uterus transplantation in the baboon: methodology and long-term function after auto-transplantation. Hum Reprod 2010;25:1980-7.

101. Mihara M, Kisu I, Hara H, Iida T, Araki J, Shim T *et al.* Uterine autotransplantation in cynomolgus macaques: the first case of pregnancy and delivery. Hum Reprod 2012;27:2332-40.

102. Scott JR, Pitkin RM, Yannone ME. Transplantation of the primate uterus. Surg Gynecol Obstet 1971;133:414-8.

103. Yonemoto RH, Du Sold WD, Deliman RM. Homotransplantation of uterus and ovaries in dogs. A preliminary report. Am J Obstet Gynecol 1969;104:1143-51.

104. Confino E, Vermesh M, Thomas W, Jr., Gleicher N. Non-vascular transplantation of the rabbit uterus. Int J Gynaecol Obstet 1986;24:321-5.

105. Sieunarine K, Doumplis D, Kuzmin E, Corless DJ, Hakim NS, Del Priore G *et al.* Uterine allotranspiantation in the rabbit model using a macrovascular patch technique. Int Surg 2008;93:288-94.

106. Johannesson L, Enskog A, Mölne J, Dahm-Kählera P, Tekin A, Diaz-Garcia C *et al.* Preclinical nonhuman primate report on allogeneic uterus transplantation Hum Reprod 2012:*in press*.

107. Kwun J, Hong X, Peng R, Szewczyk JW, Sullivan KA, DeMartino J *et al.* Unaltered graft survival and intragraft lymphocytes infiltration in the cardiac allograft of Cxcr3-/- mouse recipients. Am J Transplant 2008;8:1593-603.

108. Addy NA, Fornasiero EF, Stevens TR, Taylor JR, Picciotto MR. Role of calcineurin in nicotine-mediated locomotor sensitization. J Neurosci 2007;27:8571-80.

109. Wu S, Divall S, Hoffman GE, Le WW, Wagner K-U, Wolfe A. Jak2 is necessary for neuroendocrine control of female reproduction. J Neurosci 2011;31:184-92.

110. Nakagami F, Morishita R, Nakagami H, Osako MK, Iwabayashi M, Taniyama Y *et al.* Estrogen attenuates vascular remodeling in Lp(a) transgenic mice. Atherosclerosis 2010;211:41-7.

111. Ramirez HA, Ramirez ER, Ramirez VZ, Ramirez Nessetti DK, Nessetti MBR, Khatamee M *et al.* Pregnancy and Outcome of Uterine Allotransplantation and Assisted Reproduction in Sheep. J Minim Invasive Gynecol 2011;18:238-45.

112. Dahm-Kahler P, Wranning C, Lundmark C, Enskog A, Molne J, Marcickiewicz J *et al.* Transplantation of the uterus in sheep: methodology and early reperfusion events. J Obstet Gynaecol Res 2008;34:784-93.

113. Wranning CA, Akhi SN, Kurlberg G, Brannstrom M. Uterus transplantation in the rat: model development, surgical learning and morphological evaluation of healing. Acta Obstet Gynecol Scand 2008;87:1239-47.

114. Wranning CA, Dahm-Kahler P, Molne J, Nilsson UA, Enskog A, Brannstrom M. Transplantation of the uterus in the sheep: oxidative stress and reperfusion injury after short-time cold storage. Fertil Steril 2008;90:817-26.

115. Wranning CA, El-Akouri RR, Lundmark C, Dahm-Kahler P, Molne J, Enskog A *et al.* Auto-transplantation of the uterus in the domestic pig (Sus scrofa): Surgical technique and early reperfusion events. J Obstet Gynaecol Res 2006;32:358-67.

116. Johannesson L, Enskog A, Dahm-Kahler P, Hanafy A, Chai DC, Mwenda JM *et al.* Uterus transplantation in a non-human primate: long-term follow-up after autologous transplantation. Hum Reprod 2012;27:1640-8.

117. Racho El-Akouri R, Wranning CA, Molne J, Kurlberg G, Brannstrom M. Pregnancy in transplanted mouse uterus after long-term cold ischaemic preservation. Hum Reprod 2003;18:2024-30.

118. Wranning CA, Marcickiewicz J, Enskog A, Dahm-Kahler P, Hanafy A, Brannstrom M. Fertility after autologous ovine uterine-tubal-ovarian transplantation by vascular anastomosis to the external iliac vessels. Hum Reprod 2010;25:1973-9.

119. Ramirez ER, Ramirez Nessetti DK, Nessetti MB, Khatamee M, Wolfson MR, Shaffer TH *et al.* Pregnancy and outcome of uterine allotransplantation and assisted reproduction in sheep. J Minim Invasive Gynecol 2011;18:238-45.

120. Johannesson L, Diaz-Garcia C, Leonhardt H, Dahm-Kahler P, Marcickiewicz J, Olausson M *et al.* Vascular pedicle lengths after hysterectomy: toward future human uterus transplantation. Obstet Gynecol 2012;119:1219-25.

121. Johannesson L, Enskog A, Dahm-Kähler P, Hanafy A, Chai DC, Mwenda JM *et al.* Uterus transplantation in a non-human primate: long-term follow-up after autologous transplantation. Hum Reprod 2012;27:1640.

122. Gauthier T, Couquet C, Cornuejols MJ, Essig M, Aubard Y, Bertin F *et al.* Uterine allotransplantation in ewes using an aortocava patch. Hum Reprod 2011;26:3028-36.

123. Gill TJ, Smith GJ, Wissler RW, Kunz HW. The Rat as an Experimental Animal. Science 1989;245:269-76.

124. Parker KE, Carter CA, Murphy G, Fabre JW. The rat RT.BM1 MHC class I cDNA shows a high level of sequence similarity to the mouse H-2T23d gene. Immunogenetics 1991;34:211-3.

125. Schorle H, Holtschke T, Hunig T, Schimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. Nature 1991;352:621-4.

126. Ma Y, Wang G. Comparison of 2 heterotopic heart transplant techniques in rats: cervical and abdominal heart. Exp Clin Transplant 2011;9:128-33.

127. Abbott CP, Lindsey ES, Creech O, Jr., Dewitt CW. A technique for heart transplantation in the rat. Arch Surg 1964;89:645-52.

128. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225-9.

129. Lee S, Willoughby WF, Smallwood CJ, Dawson A, Orloff MJ. Heterotopic heart and lung transplantation in the rat. Am J Pathol 1970;59:279-98.

130. Herr NG. Orthotopic transplantation of the rat heart and lungs. Bull N Y Acad Med 1971;47:1227.

131. Arai M, Mochida S, Ohno A, Arai S, Fujiwara K. Selective bowel decontamination of recipients for prevention against liver injury following orthotopic liver transplantation: evaluation with rat models. Hepatology 1998;27:123-7.

132. Galvao FH, Waisberg DR, De Mello Vianna RM, De Castro Galvao R, Seid VE, Andraus W *et al.* Intestinal transplantation including anorectal segment in the rat. Microsurgery 2012;32:77-9.

133. Hogan B. Manipulating the mouse embryo: a laboratory manual. Plainview, N.Y: Cold Spring Harbor Laboratory Press, 1994.

134. Martínez-Gómez M, Cruz Y, Pacheco P, Aguilar-Roblero R, Hudson R. The Sensory But Not Muscular Pelvic Nerve Branch Is Necessary for Parturition in the Rat. Physiol Behav 1998;63:929-32.

135.Lehr EJ, Rayat GR, Desai LS, Coe JY, Korbutt GS, Ross DB. Inbred or outbred? An evaluation of the functional allogenicity of farm sheep used in cardiac valve studies. J Thorac Cardiovasc Surg 2006;132:1156-61.