Allogeneic ovarian transplantation using immunomodulator preimplantation factor (PIF) as monotherapy restored ovarian function in olive baboon

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Abstract

Purpose Allogeneic ovarian transplantation may be an alternative in the future to oocyte donation in women with premature ovarian failure. The objectives of this study were to (a) evaluate allotransplantation feasibility for restoration of ovarian function and (b) assess efficacy of synthetic preimplantation factor (PIF) monotherapy as sole immune-acceptance regimen.

Methods This is an experimental animal study using non-human primates (*Papio anubis*). Allogeneic orthotopic ovarian tissue transplantation was performed in two female olive baboons. PIF was administered as a monotherapy to prevent immune rejection and achieve transplant maintenance and function. Subjects underwent bilateral oophorectomy followed by cross-transplantation of prepared ovarian cortex. Postoperatively, subjects were monitored for clinical and

biochemical signs of graft rejection and return of function. Weekly blood samples were obtained to monitor graft acceptance and endocrine function restoration.

Results Postoperatively, there were no clinical signs of rejection. Laboratory parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine) did not indicate organ rejection at any stage of the experiment. Initially, significant loss of follicles was noticed after grafting and serum follicle-stimulating hormone (FSH) and E2 levels were consistent with ovarian failure. Seven months after transplantation, one animal exhibited recurrence of ovarian endocrine function (perineal swelling, E2 rise, FSH decrease, and return of menstruation).

Conclusions Organ rejection after allogeneic ovarian transplantation was prevented using PIF as monotherapy for the first time and no side effects were recorded. The study suggests the clinical feasibility of ovarian allotransplantation to obtain ovarian function.

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Introduction

Advances in cancer therapy have markedly improved survival rates, which resulted in an increased number of young cancer survivors of reproductive age. Unfortunately, high-dose cytotoxic chemotherapy and/or ionized radiation often impairs fertility and frequently leads to primary ovarian insufficiency (POI) [1]. The POI not only affects fertility but also impairs physical and psychological well-being. As a possible treatment for gonadotoxic agent-induced POI, cryopreservation

of ovarian tissue followed by transplantation has been lately used [2]. However, in women who already underwent cytotoxic treatment or who suffer from idiopathic or genetic-related POI, egg donation is the only current option for achieving pregnancy. This procedure, however, is prohibited in many countries and requires IVF, thus eliminating the chances of natural conception.

An alternative approach for addressing POI is by performing allogeneic ovarian transplantation (AOT). Although several studies investigated AOT, the use of high-dose immunosuppressants remains as the main barrier [3–5]. These drugs are associated with serious side effects leading to diabetes, hypertension, increased susceptibility to infections, malignancies, and nephrotoxicity [6]. For non-life saving transplant such as with the ovary, the toxicity of current drugs is hard to justify. Although AOT with standard immunosuppressants has been attempted in few patients, no information on long-term outcome including pregnancy is available [7, 8].

Pregnancy is a unique immune milieu, where semiallogeneic embryos or fully allogeneic embryos (in cases of ovum donation) can successfully survive and thrive, i.e., achieve both immune modulation/adaptation and transplant acceptance [9]. Identification of a pregnancy-specific responsible compound involved could revolutionize the field of transplantation by promoting tolerance, preserving anti-pathogen activity while avoiding the deleterious immune suppression.

The preimplantation factor (PIF), a peptide secreted by viable embryos, is a promising therapeutic candidate in many areas [10–13]. Established functions of this peptide include immune modulation without suppression and transplant acceptance without rejection. PIF is secreted from the two-cell stage onwards by mammalian embryos, and levels in early maternal circulation correlate with favorable pregnancy outcome [14, 15]. Immune regulatory features of PIF were successfully transposed to non-pregnant immune disorders using synthetic PIF (same sequence as endogenous PIF) [11]. PIF is effective in diverse clinically relevant autoimmunity, ionic radiation, vascular inflammation, neuroprotection, and transplant acceptance models [16–21].

Especially, short-term low-dose PIF prevented deleterious graft vs. host disease (GVHD) development following semiallogeneic or importantly allogeneic bone marrow transplantation (BMT) [22]. While GVHD was reduced, the beneficial graft vs. leukemia effect was preserved, reflecting maintained immune function. In addition, the reduced skin, liver, and colon damage [23, 24] was coupled with decreased systemic inflammation [25]. The high safety and tolerability profiles of PIF in humans have been established in the FDA-awarded FAST-TRACK, university-sponsored phase 1a/b first-in-human clinical trial for autoimmune disease (Clinicaltrials.gov, NCT02239562).

The current study aimed to evaluate PIF monotherapy used as a sole immunomodulator without any immunosuppressants to achieve allogeneic ovarian transplant acceptance in conjunction with restoration of ovarian function in a primate model.

Materials and methods

Animals used for allotransplantation

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Primate Research (IPR), Karen, Kenya (ref.: IRC/10/14). Two female (8 years old), regularly cycling healthy olive baboons (*Papio anubis*) were obtained for this study. Subjects were housed in individual cages and kept on a diet of commercially available monkey chow (Unga Feeds Ltd., Nairobi, Kenya) supplemented with vegetables, fruits, and water ad libitum [26].

Ovariectomy

For surgery, anesthesia induction was performed with 200 mg of ketamine (Agraket®, Agrar Holland BV, Soest, Holland) combined with 10 mg xylazine (Ilium Xylazil®, Troy Lab Pty Limited, Smithfield, Australia) i.m. After local lidocaine (Xylocain) spray orally, the animals were intubated. Anesthesia was maintained using halothane to minimal alveolar concentration of > 1.3 and an oxygen/nitrous oxide mixture as described before [27]. Before skin incision, each animal received a single dose of gentamycin (Vetgenta, Dawa Ltd, Nairobi, Kenya) 100 mg i.v. Through a small, subumbilical midline incision, the ovaries were identified and ovarian vessels were ligated with 4.0 glycolide and epsilon-caprolactone monofilament sutures (Monocryl®, Ethicon, Inc., Somerville, NJ, USA). Both ovaries were resected after stepwise ligation of the mesoovarian tissue and the ovarian ligament with 4.0 monofilament sutures.

Preparation of ovarian cortex

The collected ovaries were bisected in the longitudinal axis to create two fairly flat halves. The ovarian medulla was dissected out using a fine scalpel and micro-surgical scissors (S&T Microsurgery, Neuhausen, Switzerland). The remaining medullary tissue was further scraped away to obtain a thin cortical tissue (1 mm thickness) (Fig. 1). Prepared cortical tissue was incubated in a medium containing 20 ml PBS (Medicago, Uppsala, Sweden), 20 mg PIF dissolved in 4 ml PBS, and 100 µl of penicillin G/streptomycin (Eagle Long-PS, Eagle vet tech co Ltd., Chungnam, Korea) for 1 h at 37 °C with 5% CO₂.

Fig. 1 Prepared thin ovarian cortical sections before transplantation

Transplantation

For transplantation, a peritoneal pocket was created beneath the fimbriae on the broad ligament of both sides. The animals received an avascular cross-transplant of whole ovarian cortex grafts from both ovaries. Each ovarian cortex ($10 \times 10 \times 1$ mm) from the other animal was placed onto the surface of the peritoneal pocket and fixated with three to four 8.0 monofilament interrupted sutures (Monocryl®, Ethicon, Inc., Somerville, NJ, USA). After confirming a good contact of grafts to the peritoneum and hemostasis, the peritoneal pocket was left open.

Postoperative recovery was uneventful and animals received analgesia according to the research site regulations with Diclofenac 1 ml/25 mg i.m. (Shandong Shenglu Pharmaceutical Co., Ltd.; Shandong, China).

Achieving immunomodulation using PIF

Synthetic PIF (purity documented by HPLC and mass spectrometry > 95%), proprietary, was produced by Biosynthesis (Lewisville, TX, USA) and was supplied by BioIncept LLC, Cherry Hill, NJ, USA.

Immunomodulation/transplant acceptance was initiated using PIF 10 mg (with 0.75 mg/kg = 10 mg dissolved in 1 ml PBS) injected twice a day s.c. starting 1 day before surgery (Fig. 2). PIF administration dose was chosen in accordance with previously published studies [22, 28, 29]. Immunomodulation/transplant acceptance regimen after ovarian transplantation was carried out for 12 weeks injecting PIF 10 mg (dissolved in 1 ml of PBS) twice daily s.c. for 3 weeks (= one treatment cycle) with 1-week break in between each

cycle (Fig. 2). Afterwards, upon observing no rejection or side effects, PIF administration was discontinued according to the protocol and subjects were followed without any therapy for an additional 6 months for a total of 9 months (Fig. 2).

Surveillance for immune acceptance (monitoring signs of rejection) and return of ovarian function

Animals were followed up closely after surgery with a weekly physical examination including blood pressure, body temperature, pulse, and respiratory rates. In addition, weight, urine output, skin, and perineum were monitored. The blood sampling was done under short anesthesia with 200 mg of ketamine combined with 10 mg xylazine i.m. Blood tests for blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were performed weekly to assess indirect signs of rejection.

Follicle-stimulating hormone (FSH) and estradiol (E2) assays were performed monthly to determine return of ovarian function. Serum FSH radioimmunoassay (RIA) was performed by the Endocrine Technologies Support Core (ETSC) at the Oregon National Primate Research Center (ONPRC; Beaverton, OR) using reagents from Dr. Albert Parlow at the National Hormone and Peptide Program (NHPP, Harbor-UCLA Medical Center, Los Angeles). This is a homologous cynomolgus macaque assay with recombinant cynomolgus FSH (AFP-6940A) for both iodination and standards and has previously been used in the ETSC to measure FSH in multiple non-human primate species including baboons. The rabbit anti-cynomolgus FSH, AFP-782594, was used at a final dilution of 1:1,038,462. The standard curve ranged between 0.005 and 10 ng/tube and the detection limit of the assay was 0.005–0.02 ng/tube. The intra-assay variation for this assay was 10.6%. Because all samples were analyzed in one assay, no inter-assay variation was calculated for these samples, but the overall inter-assay variation for this assay in the ETSC is less than 15%.

Estradiol (E2) levels were analyzed by immunoassay using a Roche cobas e411 automated clinical platform (Roche Diagnostics, Indianapolis, IN) at the ETSC. This assay was previously validated for use in non-human primates [30, 31]. The assay sensitivity range for the E2 assay was 5–3000 pg/ml. Intra- and inter-assay CVs for the Roche assays are consistently less than 7%.

Return of ovarian function was confirmed when perineal swelling followed by menstruation (representing typical signs of cyclicity in this species) was observed (Fig. 3) [32]. Euthanasia was conducted under general anesthesia with 200 mg of ketamine (Agrakert®, Agrar Holland BV, Soest, Holland) combined with 10 mg xylazine (Ilium Xylazil®, Troy Lab Pty Limited, Smithfield, Australia) i.m. followed by intravenous injection of 100 mg/kg of 20% sodium pentobarbital (Sigma-Aldrich, Taufkirchen, Germany).

Fig. 2 PIF monotherapy as transplantation preconditioning and maintenance. Each baboon was preconditioned with PIF prior to ovariectomy and received PIF Rx bid (3 weeks on, 1 week off) for

12 weeks as transplant maintenance. Both baboons were monitored for additional 6 months without any added treatment. Return to function (menstruation) was documented in one subject at week 42

Histology to assess ovarian tissue status before and after allografting

Ovarian biopsies collected shortly before transplantation and after euthanasia were fixed in 10% formaldehyde and dehydrated progressively in increasing concentrations of ethyl alcohol (50, 70, 80, 90%, and absolute alcohol), after which they were immersed in toluene. Subsequently, they were processed through toluene three times and then infiltrated in a paraffin wax mixture in an oven (Oven model-5831, National appliance Co., Portland, Oregon, USA). The tissues were then oriented in a perpendicular fashion on a piece of embedding ring and embedded in molten wax. For histological evaluation, paraffin-embedded tissue was processed as 5- μ m sections, deparaffinized, and stained with hematoxylin–eosin (HE). The sections were stained with harris hematoxylin for 7 min then washed in running tap water before decolorizing in 0.5% acid alcohol and toning in ammonia water. Toned sections were counterstained with eosin Y for 5 min then washed in water. Stained sections were then dehydrated through several changes of increasing concentrations of ethanol, cleared in xylene, and finally mounted using DPX.

Histology sections were analyzed in a Leica DM500 Microscope (Leica Microsystems Ltd., Heerburgg, Switzerland) and photo documentation was performed with a Leica ICC50 Camera (Leica Microsystems Ltd., Heerburgg, Switzerland).

Fig. 3 A non-inflated perineum post-transplantation on the left and an inflated perineum as a clear sign of estrus on the right

Results

Clinical and laboratory parameters to assess transplant immune acceptance with PIF monotherapy as sole maintenance regimen

Postoperative recovery was uneventful in both animals. No clinical or laboratory signs of rejection were observed at any time throughout the 9 months of follow-up. Scarless wound healing was observed within the first 16 weeks, and by week 31, regrowth of local hair was documented (Fig. 4). Biochemical parameters revealed no major deviations from normal levels in kidney and liver function parameters that would indicate rejection (Fig. 5). Clinical parameters for both animals remained stable throughout the 9-month observation period (i.e., 3 months of treatment and 6 months of post-treatment observation) with no clinical signs of rejection.

Endocrine assays to evaluate the return of ovarian function

Both animals showed a continuous decline of FSH levels after initially showing high FSH values consistent with ovarian failure immediately postoperatively (Fig. 6). Animal number 1 showed a decline of 39.33% and animal number 2 a decline of 29.27% in FSH levels within the first 7 months postoperatively. Animal number 1, with the more distinct FSH decline, had a 29% lower mean FSH than animal number 2. Estradiol

Fig. 4 Scarless wound healing with regrowth of hair during PIF therapy

levels remained undetectable for the first 7 months after transplantation.

A rise in estradiol levels (28.73 pg/ml) in animal number 1 was documented 229 days after ovarian transplantation following perineal inflation and an episode of menstruation (Fig. 3). Thereafter, no further signs of cyclicity were detected and, at 324 days after transplantation, the animals were euthanized for autopsy.

Gross morphology and histology of ovarian grafts

Pre-transplant histology showed intact ovarian tissue with multiple follicles at different stages in both animals. Macroscopically antral follicles and corpus luteum cysts were observed. When ovarian grafts were inspected in situ, there was no sign of rejection macroscopically (Fig. 7). However,

post-transplantation histology revealed a significant depletion of follicles.

Discussion

Herein, we report ovarian allotransplantation in a primate model without encountered signs of rejection followed by recurrence of endocrine function. We show that PIF, an immune modulatory synthetic peptide monotherapy, achieved long-term transplant acceptance after stopping therapy, which led to restored ovarian endocrine function in a primate model. Importantly, PIF treatment throughout the treatment period and for several months afterwards was not associated with side effects or any apparent signs of rejection, supporting its use for ovarian allotransplantation and beyond.

Fig. 5 Illustrating post-transplantation biochemical (a) and clinical (b) parameters. Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, Temp = temperature, Resp = respiration

Fig. 6 FSH levels after transplantation

Over the last decade, growing attention has been paid to the field of gynecological organ transplantation. Ovarian autotransplantation to restore fertility after cancer treatment is slowly moving from an experimental procedure towards a routine clinical strategy, with more than 100 children born worldwide to date. However, due to variability in the graft survival, further improvements are required to enable sustained long-term ovarian function [2, 33]. Recently, first successful cases of uterine allotransplantation resulted in several live births [34, 35]. However, due to the non-lifesaving character of the procedure, the use of immunosuppression in uterine allotransplantation remains highly controversial [34]. Ovarian allotransplantation combined with classical immunosuppressive agents would encounter similar medical and ethical challenges. The ovary is not an immunologically

Fig. 7 Appearance of ovarian grafts in situ 324 days after transplantation

privileged organ unlike some authors suggested in the past. Indeed, most research showed aggressive immunorejection in animal models of ovarian allogeneic transplantation, when immunosuppressive drugs were not utilized [36–38]. Even with high-dose cyclosporine, allotransplants failed to survive and were rejected [36]. In mice that had undergone allogeneic ovarian transplantation with no immunosuppression, bioluminescence methodology demonstrated a rapid loss of ovarian function and aggressive organ rejection [39]. Furthermore, low CD4+/CD8+ ratio of peripheral T-cells with high CD4+/CD8+ cells infiltration into the ovarian allograft confirmed aggressive immune rejection after allotransplantation [40].

Ovarian allotransplantation could serve as a potential cure for women suffering from POI. Etiology of POI includes gonadotoxic cancer treatment, benign surgery (e.g., endometriosis), genetic disorders (e.g., Turner syndrome), and idiopathic POI [41]. Besides infertility, patients with POI are at increased risk for cardiovascular disease and osteoporosis, leading to reduced life expectancy, if not treated properly [42–44]. Various POI manifestations like infertility and vasomotor symptoms impair the quality of life and psychological well-being [45]. Oocyte donation is a reasonable option to achieve pregnancy in patients with POI, and several well-tolerated medications are available to manage the side effects of hormone deprivation [46–48]. Established immunosuppressants, such as glucocorticoids and the calcineurin inhibitors cyclosporine and tacrolimus as well as the antiproliferative agents azathioprine and mycophenolate mofetil, are associated with serious side effects like nephrotoxicity, hypertension, diabetes, malignancies, and infections [6]. As the ovary is not a vital organ and when considering known risks of rejection and the toxicity of immunosuppressants, clinical application of AOT can be a very controversial issue.

Nevertheless, AOT is a potentially useful and powerful tool not only to restore fertility but more importantly to restore endocrine function in women suffering from POI, provided that the organ rejection can be prevented by using simple to administer and non-toxic agents or methods. Ideal transplant regimens would be safe (devoid of deleterious side effects), assure acceptance for the long term (obviate need for transplant removal), obviate the need for organ matching, and facilitate/enable restoration of organ functionality (menstrual cyclicity and/or even pregnancy).

The search for safe and effective immune regulatory compound(s) that would lead to allograft acceptance without immune suppression and restore function is a long-term quest. In this study, PIF immunomodulator was used and shown to prevent organ rejection and lead to organ function. Endogenous PIF is an evolutionarily conserved mammalian peptide secreted by viable embryos and present in maternal circulation of viable pregnancies [10, 11, 14, 15, 49, 50]. Synthetic PIF replicates endogenous PIF's function and is

effective in several non-pregnant preclinical models, acting in integrated manner locally (target organ) and on the systemic immunity. Despite short circulating half-life, PIF exerts long-term pharmacodynamic effects after being cleared from circulation [16]. Such a delayed beneficial effect of PIF monotherapy following semi/allogeneic bone marrow transplant and after lethal total body irradiation was already reported [22–24]. PIF targets CD14+ cells, namely antigen-presenting cells (APC) shifting them from effector to regulatory phenotype. Through upregulation of B7H1 expression, T-cell response is redirected to repair instead of inflammation. PIF reduces NK cell cytotoxicity by decreasing CD69 expression—a possible contribution to the observed organ acceptance [51]. Notably, CD69, an inducer of T-cell activation, is increased in acute organ rejection in renal and heart transplant patients [52–54]. Collectively, borrowing from embryo/maternal interaction, PIF selective action on innate and adaptive immunity “at need” enables this critical balance between tolerance/rejection and maintains immune response to danger signals.

In this study, PIF monotherapy was administered as the sole transplant maintenance regimen (no steroids or any other drugs administered) and used at three different settings. First, the donor/recipient was injected with PIF prior to transplant with the goal of achieving recipients’ immune homeostasis, reducing surgery-induced inflammatory response. Second, ovaries were preconditioned with PIF *ex vivo* in between ovarian harvesting and transplantation in short-term culture to reduce inflammation and possibly to preserve functionality prior to transplant, as reported for allogeneic stem cells [22]. Third, following the successful transplant, PIF was administered (in a physiological dose range) for 3 weeks on and 1 week off for three consecutive months to regulate immune response and decrease vascular inflammation and oxidative stress to support transplant acceptance without the need of an immune suppressor [22–24]. It is important to differentiate PIF’s action from anti-inflammatory agent, since inflammation is necessary for proper healing. This is supported since the laparotomy scar healed completely and restored hair growth during PIF administration. Whether the graft’s local acceptance was aided is unknown at present.

We designed the study with a baboon model due to its similarity to humans in anatomy and menstruation pattern. Baboon menstrual cycle is 33.4 ± 2.1 days with the duration of 3.2 ± 1.0 days [55]. Furthermore, its cyclic hormonal activities can be very easily assessed by perineal inflation [26, 55–58]. Studies on uterus and kidney transplantation indicate that baboons have a similar rejection pattern as in humans [26, 59], enabling the current study on AOT. The present study represents the first experiment on allogeneic ovarian transplantation in this species. Thus, no data is available on AOT in baboons including the natural course of AOT. Monitoring rejection following AOT is difficult, since organ biopsy

cannot be performed due to the small size and location of the grafts and systemic immunological markers for rejection are not well validated in baboons. Herein, rejection was assessed by changes of clinical signs (skin, perineum, vital signs, behavior, and weight) as well as in laboratory tests (serum markers for liver and kidney function). Since the goal of this study was restoration of ovarian function and fertility, we did not test systemic immunological markers. Instead, we focused on monitoring endocrine function with serum FSH and E2 levels and return of menstruation as a proof of successful immunologic acceptance. Since the animals used for this study are wild-caught, genetic relativity cannot be ruled out completely. Randomized controlled animal studies will be helpful to gain more knowledge and feasibility on AOT by assessing the natural course of AOT, validating immunological rejection parameters and testing genetic compatibility in the observed subjects.

In addition to transplant acceptance without side effects, we demonstrated return of ovarian function after AOT in one animal, evidenced by increased E2, perineal inflation, and menstruation, albeit only for a short period. The histology of recovered ovarian grafts (from both animals) at the time of autopsy revealed significant loss of follicles. Mechanisms involved in follicular loss after transplantation are complex (but most important factor can be ischemia [60]), which could further be augmented by the silent immune rejection.

In our view, live donors will be the main source of donated ovarian grafts for future human AOT due to paired character of ovaries and the relatively low-risk laparoscopic surgery to perform unilateral oophorectomy. The use of posthumous organ donors for AOT could also be a viable option, but it may raise ethical concerns [61]. To improve the efficacy and prevent misuse of this technology, it may be necessary to establish ovarian donor selection criteria such as age and ovarian reserve.

In summary, we demonstrated the first ovarian allotransplantation using PIF monotherapy in a non-human primate model, which resulted in transplant acceptance and recurrence of ovarian endocrine function without the use of any immunosuppression or any other treatment. The study opens the door to using PIF as acceptance and maintenance regimen in organ transplantation. Further studies are required to assess the efficacy of AOT using PIF treatment in women.

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