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ORIGINAL ARTICLE

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

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STUDY QUESTION: Do therapeutic levels of cyclosporine-A and tacrolimus affect ovulation in a rat gonadotrophin-induced ovulation model?

SUMMARY ANSWER: Cyclosporine-A, but not tacrolimus, decreases ovulation rate when administered for 5 days before induced ovulation.

WHAT IS KNOWN ALREADY: The mainstays of immunosuppression in solid organ transplantation, to prevent rejection, are the calcineurin inhibitors cyclosporine-A or tacrolimus. These drugs could potentially affect fertility in transplanted patients. Since ovulation is an inflammation-like process with pivotal roles for several immune cells and modulators, it is possible that the calcineurin inhibitors, with broad effects on the immune system, could interfere with this sensitive, biological process.

STUDY DESIGN, SIZE, DURATION: Experimental design at university-based animal facilities. A total of 45 immature Sprague–Dawley rats were used. The study was carried out over 3 months.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Immature Sprague–Dawley rats (n = 45) were randomly assigned to receive equivalent doses of tacrolimus (0.5 mg/kg/day; TAC), cyclosporine-A (10 mg/kg/day; CyA) or vehicle (Control). Ovarian hyperstimulation was induced with 10 IU of equine chorionic gonadotrophin, and ovulation was triggered with 10 IU of hCG. Oocytes were retrieved from the oviducts and ovulation rates were calculated. Various subpopulations of white blood cells were counted in peripheral blood and ovarian tissue samples.

MAIN RESULTS AND THE ROLE OF CHANCE: Animals in the CyA group showed a lower ovulation rate when compared to the TAC and Control groups (CyA: mean 9 oocytes (range 0–22); TAC: 21 oocytes (8–41); Control: 22 oocytes (6–39); $P = 0.03$). Regarding counts of the white blood cell subpopulations and resident neutrophils in the ovary, no significant differences were observed between the groups.

LIMITATIONS, REASONS FOR CAUTION: Although the ovulation process is highly conserved within species, the differences between rodents and humans may limit the external translatability of the study.

WIDER IMPLICATIONS OF THE FINDINGS: These findings suggest that tacrolimus should be the preferred calcineurin inhibitor of choice in transplanted patients who are aiming for pregnancy.

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Key words: cyclosporine-A / calcineurin / immunosuppression / ovulation / ovary / rat / tacrolimus / transplantation

WHAT DOES THIS MEAN FOR PATIENTS?

'Calcineurin inhibitors' are specific drugs used to prevent rejection after organ transplantation. Cyclosporine-A and tacrolimus are two main drugs currently used.

Ovulation could be considered an inflammatory process in which cells of the immune system would participate. In this way, several inflammatory processes could be affected by the immunosuppressive effects of calcineurin inhibitors and so ovulation could be disrupted in patients taking these medicines. This study was designed to look at whether the giving women cyclosporine-A or tacrolimus alters ovulation, and therefore, the natural fertility in female recipients of solid organ transplants.

Introduction

The use of allogeneic transplantation of solid organs and composite vascularized tissues in the treatments of life-threatening diseases or to compensate for loss of body function has increased tremendously during the last decades. The introduction of the calcineurin inhibitor cyclosporine-A (CyA) in the 1980s increased the success rate of solid organ transplantation. This fact contributed to the expansion of transplantation of non-life saving organs/tissues, such as the hand, face, intestine and pancreas. In the 1990s, a new calcineurin inhibitor with a less nephrotoxic side effect profile, tacrolimus (TAC), was introduced (Busuttil *et al.*, 1994).

In parallel with the expansion of the patient groups that are treated with transplantation, the general survival of the patients and general health of the transplanted patients have improved considerably. The medical focus on the transplanted patient has also, apart from graft survival with restored organ function, come to include several issues related to quality of life, with fertility being one important issue. Thus, by the year 2006 more than 14 000 births had been reported since the first post-transplantation pregnancy, which took place more than 50 years ago (McKay and Josephson, 2006). Although the rate of obstetric complications seems to be increased among immunosuppressed women carrying transplants, the risk of fetal malformation is similar to that of the normal population (McKay and Josephson, 2006).

The effects of transplantation on subsequent fertility are poorly understood. A recovery of female fertility after organ transplantation has been widely described in the literature (Douglas *et al.*, 2007), but this has been linked to the considerable improvement of the woman's health when she is changing from a status with end-stage disease into one with restored and normal organ function. There may exist negative effects of the immunosuppressive drugs on the function of the reproductive organs. Animal studies in rodents suggest that the implantation rate is decreased and the miscarriage rate is increased as a result of exposure to cyclosporine (Mason *et al.*, 1985; Groth *et al.*, 2010). There also exist case reports on affected ovarian function after organ transplantation in women (Cure *et al.*, 2004).

Ovulation is a complex biochemical process that lasts around 36 h in humans and approximately 12 h in rodent species. During this ovulatory process, structural and functional changes occur, and these allow the release of a fertilizable oocyte and the transformation of the follicle into a corpus luteum. Several mediator pathways induce changes in the follicle that are necessary for follicular rupture. Breakdown of the extracellular matrix on the follicular apex (Li and Curry, 2009) and expansion of the extracellular matrix around the cumulus cells (Eppig, 1982) seem to be the most important components for normal ovulation to occur. These processes bear many similarities to an inflammatory reaction, with participation of classical inflammatory mediators, such as cytokines, collagenases, stromelysins, gelatinases, and prostaglandins, as well as pivotal roles for certain leukocyte subsets (Brännström *et al.*, 1994; Suzuki *et al.*, 1998). Several of these inflammatory pathways could potentially be affected by the immunosuppressive effects of calcineurin inhibitors (Garcia-Criado *et al.*, 1997).

Recent successful attempts of human uterus transplantation have further shed light on the issue of possible effects of immunosuppressant drugs on ovarian function (Brännström *et al.*, 2015). Since this is the first type of allogeneic transplantation where restored fertility is the goal, with menstruation being the first obvious sign of normal ovarian function, it is important to ascertain that ovarian function takes place normally (Brännström *et al.*, 2015). To our knowledge, there exist no systematic research studies on the effects of immunosuppressant drugs on ovarian function after any type of solid organ transplantation. The main type of immunosuppressant in all immunosuppressant protocols is the calcineurin inhibitor, with either cyclosporine-A or the more modern drug tacrolimus being the drug of choice. In the present study, we aimed to evaluate for the first time in any species the effects of the calcineurin inhibitors on the ovulatory process. To reach this objective, we examined whether therapeutic levels of cyclosporine-A and tacrolimus affect ovulation in a murine gonadotrophin-induced ovulation model.

Materials and methods

Experimental design and animals

Immature Sprague–Dawley rats (Harlan, Horst, Netherlands) were used in this experiment. The animals arrived at our facilities at the age of 16 days and were then housed in controlled conditions (21–23°C; illumination 0700–1900 h) and had access to water and food *ad libitum*. At the age of 21 days, all animals were weighed and then randomly assigned to three different groups (15 animals/group): Group 1—0.9% NaCl (Control); Group 2—cyclosporine-A 10 mg/kg/day (CyA); and Group 3—tacrolimus 0.5 mg/kg/day (TAC). Although a 20-fold difference in doses is needed to elicit the immunosuppressive effect of the two calcineurin inhibitors, tacrolimus presents comparable effects to cyclosporine-A (Barbarino *et al.*, 2013).

All animals were synchronized for ovulation at 25 days of age, according to our standardized protocol for ovulation studies (Brännström *et al.*, 1995). Briefly, rats aged 25 days were injected s.c. with 10 IU of equine chorionic gonadotrophin (eCG) at 12:00 a.m. Forty-eight hours later, the rats were administered with 10 IU of hCG

(both purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden), with ovulation occurring 10–15 h after hCG administration (Espey *et al.*, 1985). Twenty hours after ovulation induction, the animals underwent euthanasia. Blood samples were then taken for white blood cell quantification and measurement of levels of calcineurin inhibitor. The ovulation rate was also assessed (see below). Both ovaries were taken for histological and molecular studies. The present study was conducted in accordance with Swedish legislation and was approved by the Animals Ethics Committee in Gothenburg. All procedures related to animals were performed by the same operator. A schema of the experimental design is shown in Fig. 1.

Administration of calcineurin inhibitors

Miniosmotic pumps (Alzet, Durec Corp, Cupertino, CA, USA) were filled with a solution of tacrolimus (diluted in NaCl-pump model 1007D) or cyclosporine-A (diluted in 1,2-propanediol -pump model 2001) prepared individually for each animal weight. Miniosmotic pumps were filled only with 0.9% NaCl for use in the control group. Two different models (1007D and 2001) of pumps, with identical external composition, were used since different concentrations of drugs were studied. The pumps were primed in NaCl at 37°C for 12 h prior to its insertion, according to the manufacturer's instructions. During the first day of intervention (postnatal Day 21), a skin incision (5 mm) was made on the back of each animal caudally to the neck, under isoflurane anesthesia. The pump was placed s.c. in a parasagittal position, and the skin subsequently closed with two polyglactin stiches (Vicryl 4-0, Ethicon, Somerville, NJ, USA). Pumps remained in animals for 7 days and then were removed at postnatal Day 28.

Calcineurin inhibitor levels

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

White blood cell subpopulations in peripheral blood

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

Assessment of ovulation rate and preservation of ovaries

The ovulation rate was assessed 20 h after injection of hCG. The oviduct was gently dissected from the ovaries and the uterine horns. The left ovary was kept in RNAlater (Qiagen GmbH, Hilden, Germany) and stored immediately at −20°C. The right ovary was kept in 4% paraformaldehyde diluted in PBS (Sigma-Aldrich, St. Louis, MO, USA). The ampullary regions of both oviducts were then isolated, placed into a petri dish with tempered Flushing Medium at 37 °C (Origio, Måløv, Denmark) and opened to release and collect the cumulus-oocyte complex. In order to scatter the oocyte-attached cumulus cells from these oocytes, they were incubated with hyaluronidase (Hyase®-10X, Vitrolife AB, Göteborg, Sweden) diluted to a ratio 1:10 with supplemented G-GAMETE™ (Vitrolife AB, Göteborg, Sweden), for 10 min over a glass microscope slide. Then, the oocytes were covered with a coverslip and they were classified according to maturational stage and counted under a Nomarski interference microscope. The total number of mature oocytes corresponded to the ovulation rate.

Expression of ELANE, MPO, TIMP3, and RUNX2

Elastase (neutrophil expressed; ELANE) and myeloperoxydase (MPO) gene expression was used for quantification of neutrophils within the tissue. Tissue inhibitor of metalloproteinase 3 (TIMP3) and runt-related transcription factor 2 (RUNX2) were used as post-ovulation molecular markers. Total RNA was isolated from the left ovary using Trizol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was made using 2 mg total RNA with 0.5 mg random primer (Promega, Madison, WI, USA) in a total volume of 17 µl. This mixture was denatured at 70°C for 5 min. Then, 0.5 mM deoxy-NTP, 20 U RNAsine, reverse transcriptase buffer and 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) were added to a final volume of 25 µl. The cDNA synthesis was performed for 60 min at 37°C. Quantitative RT-PCR in an ABI Prism 7000 Sequence Detector (Applied Biosystems, Carlsbad, CA, USA) was performed to evaluate the mRNA expression levels. Commercially available Taqman MGB probes (Applied Biosystems, Carlsbad, CA, USA) were used for the target genes TIMP3 (ID: Rn00441826_m1), RUNX2 (ID: Rn01512298_m1), ELANE (ID: Rn01535456_g1), MPO (ID: Rn01460205_m1), and control (β-actin) gene (ID: Rn01424440_s1). Each amplification reaction consisted of 20 ng cDNA, 1x probe-mix and 1x TaqMan Universal PCR mastermix (Applied Biosystems, Carlsbad, CA, USA) in a final volume of 25 µl. After controlling for the amplification efficiency of the target genes and of the control, the relative expression was determined using the comparative Ct method. Expression level of the target gene mRNA was normalized to the expression of the control (β-actin).

Histology

The right ovary was kept in 4% buffered formaldehyde, then dehydrated, embedded in paraffin and cut into 5 µm thick sections. Sections were subsequently deparaffinized and rehydrated through graded ethanol, rinsed in distilled water and treated with 0.3% H_2O_2 and 10% normal horse serum to block endogenous peroxidase and non-specific binding, respectively. Primary antibodies against CD4 (IgG

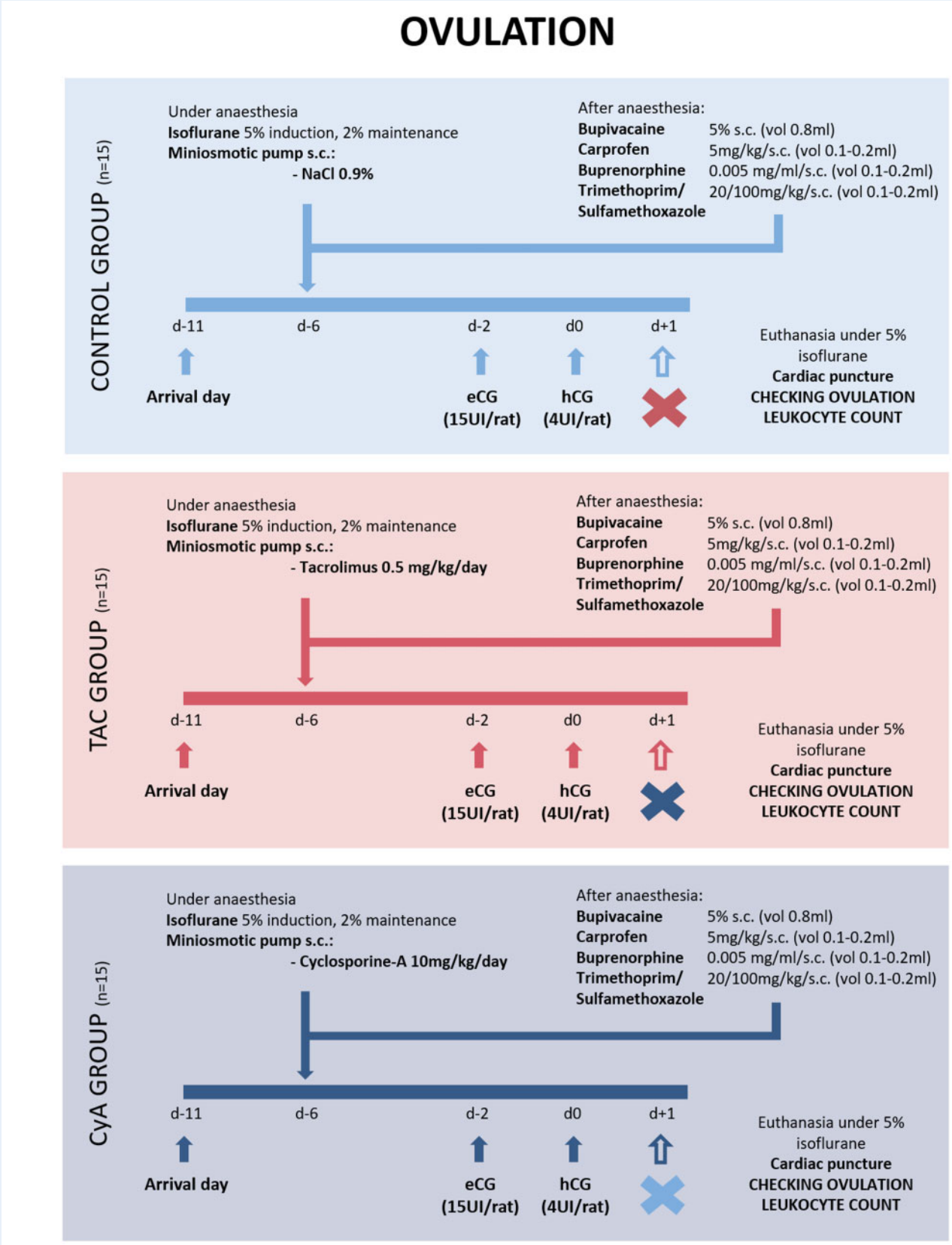

Figure 1. Scheme of the experimental design using a rat gonadotrophin-induced ovulation model. Sprague–Dawley rats were synchronized for ovulation by injecting 10 IU of equine chorionic gonadotrophin (eCG) s.c.; 48 h later, ovulation was induced by injecting 10 IU of hCG i.p. and 20 h after induction, the animals were euthanized by cervical dislocation. During the 7 days prior to euthanasia, the animals received tacrolimus 0.5 mg/kg/day (TAC; n = 15); cyclosporin-A 10 mg/kg/day (CyA; n = 15); or normal saline (Control; n = 15).

concentration 1.0 mg/ml; dilution used for immunoassays 1:100, Abbiotec 250592, Nordic Biosite, Täby, Sweden), CD8 (IgG concentration 1.0 mg/ml; dilution 1:75; Serotec MCA48R, Nordic Biosite, Täby, Sweden), CD163 (IgG concentration 0.5 mg/ml; dilution 1:50; Serotec MCA 342 R, Nordic Biosite, Täby, Sweden), and CD25 (IgG concentration 1.0 mg/ml; dilution 1:25; Serotec MCA 273 R, Nordic Biosite, Täby, Sweden) were incubated on sections at room temperature for 60 min.

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

Statistical analysis

Normal distribution of the data was tested using the Kolmogrov–Smirnoff test. Normally distributed data were presented as means and SD. Data that were not distributed normally were presented as medians and ranges (between square brackets). Pairwise comparisons between the experimental groups and the control group were performed using Student's *t*-test or the Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered as statistically significant. Calculations were carried out using SPSS v 20 (IBM Corp, Chicago, IL, USA). The number of animals to be included in the study was estimated through G*Power 3.1.9.2 software (Franz Faul, Universität Kiel, Germany). The specific statistical t-test calculated the difference between two independent means (Control vs. CyA or TAC). The type of power analysis used was *a priori* and the input parameters were two tails, an effect size for ovulation rate = 0.9, α error probability = 0.05, β error probability = 0.34 and allocation ratio N2/N1.

Results

Calcineurin inhibitors levels

The median plasma levels of cyclosporine-A and tacrolimus were measured after obtaining samples from aorta at euthanasia. The results were 2128 ng/ml (1578–2892) and 5.5 ng/ml (4.6–6.6), respectively. Cyclosporine-A and tacrolimus levels in the control group were undetectable.

White blood cell counts

White blood cell counts are shown in Table I. Eight samples/group were analyzed. The rest of the samples were not counted because of experimental errors in obtaining blood samples from aorta during euthanasia due to blood clotting. No significant differences were found between the three groups for the white blood cell counts. However, there was a significant decrease in the number of lymphocytes in the CyA group compared to Control ($P<0.05$). The number of monocytes and eosinophils was similar between experimental groups (CyA and TAC), but significantly different from the Control group. Therefore, the use of calcineurin inhibitors slightly modified the different white blood cell subpopulations.

Table I Counts of total white blood cells and their subpopulations in rat peripheral blood.

	Control (n = 8)	CyA (n = 8)	TAC (n = 8)	*P*-value
WBC (cells/µl)	3063 ± 1560	3178 ± 1246	1958 ± 645	n.s.
Lymphocytes (%)	86 ± 3	77 ± 6	85 ± 6	[a]$P<0.05$
Neutrophils (%)	10 ± 4	13 ± 3	8 ± 6	n.s.
Monocytes (%)	3 ± 1	7 ± 5	5 ± 3	[a]$P<0.05$
Eosinophils (%)	0 [0–1]	0 [0–1]	0 [0–2]	[b]$P<0.05$
Basophils (%)	0 [0–1]	0	0	n.s.

WBC, white blood cells; CyA, cyclosporine-A; TAC, tacrolimus.
Comparisons were performed using the Control group (normal saline) as reference. Student's *t*-test was used. Results are expressed as mean ± SD. Differences were considered significant if $P<0.05$.
[a]Significant differences between Control and CyA group.
[b]Significant differences between Control and TAC.

Ovulation rate

Animals in the cyclosporine group showed a decreased number (9 (0–22), $P=0.03$) of ovulated oocytes when compared to the control group (22 oocytes (6–39), Fig. 2). No significant difference was found between control and the tacrolimus-treated group (21 (8–41)).

Ovarian neutrophilic markers and ovulation markers

The MPO mRNA expression level was significantly decreased in the TAC- and CyA-groups when compared to the control group ($P=0.019$). No differences between groups were found regarding ELANE mRNA expression. Post-ovulatory status and anti-proteolytic activity, assessed by measuring RUNX2 and TIMP3 mRNA levels, respectively, were not significantly different between groups, although RUNX2 mRNA expression level in TAC group ovaries tended to be downregulated. All RT-PCR results are shown in Fig. 3.

Histology

Antibodies against CD163 recognize a specific surface glycoprotein present in macrophages of most kinds of tissue, although it does not bind to monocytes. Two main patterns of CD163+ cells were identified in ovaries from this experiment: perivascular (Fig. 4A) and within the newly formed corpus luteum (Fig. 4B). Such a distribution was reproduced in all experimental groups. The total amount of CD163+ cells/corpus luteum did not differ between groups (Control: 14.9 ± 6.7; TAC: 16.2 ± 8.2; CyA: 14.7 ± 5.2; n.s.). CD4 is mainly expressed by T-helper lymphocytes. CD4+ cells were absent in all samples but two (one control and one TAC). In those samples with CD4+ cells, its density was low (0.4 and 0.3 cells/corpus luteum,

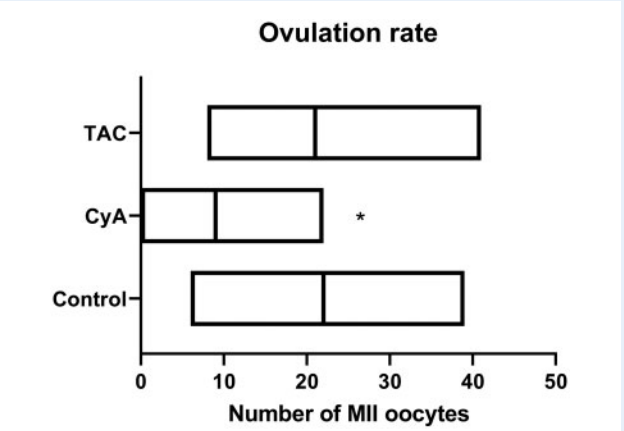

Figure 2. Ovulation rate outcomes in the rat gonadotrophin-induced ovulation model. Bars represent the median (vertical line) and ranges for the ovulation rate in different groups. Significant differences between Control and CyA group are represented by * ($P<0.05$). MII: metaphase II.

respectively). The CD8 co-receptor is predominantly expressed on the surface of cytotoxic T cells. CD8+ cells were present in all groups and no statistical differences were seen between groups (Control: 5.1 ± 3.2 cells/corpus luteum; TAC: 6.2 ± 2.9 cells/corpus luteum; CyA: 7.1 ± 4.0 cells/corpus luteum; n.s.).

Discussion

The greatest development in transplantation surgery was the introduction of effective immunosuppression by the calcineurin inhibitor cyclosporine-A in the 1980s and tacrolimus around 15 years later (Busuttil *et al.*, 1994). Since then, there has been a continuous increased utilization of transplantation as treatment of severely compromised organ function, with kidneys being the most prevalent transplanted organ. Moreover, during recent years, vascularized composite tissue transplantation, such as transplantation of the hand and face, has reached the stage of clinical routine procedures. Today around 18% of transplanted women are of reproductive age (according to the Organ Procurement and Transplantation Network—OPTN—data as of 31 August 2014) and, with the continuously increasing graft survival, the issue of forming a family is a central concern for the younger age groups of patients, that usually return to leading normal lives after transplantation. It is generally perceived that the fertility potential of a female on immunosuppressive medication is compromised although no systematic studies have been carried out on the subject. There are human case reports and a few research studies in animal models (El-Akouri *et al.*, 2006; Groth *et al.*, 2009), which could indicate that the calcineurin inhibitors decrease implantation rate and increase miscarriage rate. These negative consequences were found in a previous study from our group where a direct cyclosporine-A exposure during pregnancy altered the endometrium adversely in a dose-dependent manner (Groth *et al.*, 2009).

To our knowledge, there is limited evidence on the effect of immunosuppressive drugs on ovarian function. A recent study performed on a mouse model of polycystic ovary syndrome (PCOS) tested the beneficial effect of low-dose tacrolimus (0.1 mg/kg) in supporting the ovarian and follicular immune system and promoting gestational success (Albaghdadi *et al.*, 2019). Here, the high-fat-fed New Zealand Obese mice (HFD-dNONcNZO) presented obesity-associated adverse reproductive outcomes in PCOS-susceptible individuals. After treatment with tacrolimus, a change in the balance of macrophages and T cells was observed, which helped to mitigate the effects of PCOS and, therefore, supported the environment for achieving gestation. The biologically central process of ovulation has been compared to a local inflammatory reaction. A number of inflammatory mediators and also some subsets of leukocytes are central to carry out the tissue remodeling of the extracellular matrix, blood flow changes and cumulus cell ultrastructural modifications, which are so important in ensuring that follicular rupture takes place. The intraovarian biochemical changes of ovulation are time- and site-specific and involve various types of molecules with antagonistic effects, essentially metalloproteinases and inhibitors of metalloproteinases, and also different type of effector cells (theca cells, granulosa, and resident leukocytes) that interact, promoting matrix remodeling. The final purpose of these changes is the extracellular digestion of the basal lamina and the surrounding extracellular stroma to allow the release of matures oocytes from follicles. In this balancing mechanism, there is a key role for metalloproteinases secreted by white blood cells in the ovary and the release of inhibitors of metalloproteinases secreted by theca cells, granulosa cells, and fibroblasts (Fedorcsak *et al.*, 2010).

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

Data in the literature regarding the use of immunosuppressants and ovulation may seem contradictory: while some studies demonstrated an improvement in fertility parameters after transplantation of solid organs (Douglas *et al.*, 2007), others described an ovarian dysfunction in solid organ-transplanted patients, usually associated with mid-luteal phase defects caused by low progesterone levels (Yildirim *et al.*, 2005).

To our knowledge, this is the first study to systematically evaluate the effect of calcineurin antagonists on ovulation. The doses used in this experiment were chosen because they result in plasma levels of calcineurin inhibitors similar to those obtained in a clinical setting of immunosuppression in solid organ transplant. These doses are efficient enough to control the risk of organ rejection in most clinical scenarios. Despite the differences between species, pharmacokinetic calcineurin inhibitor profiles in mouse and human models are quite similar, , as is their pharmacodynamic profile. Our main finding is that cyclosporine-A, but not tacrolimus, decreases ovulation rate when administered for a period of 5 days before induced ovulation. These differential effects on ovulation by the two types of calcineurin inhibitors were unexpected when we planned the experiment, because the main mechanism of action of both is the specific inhibition of calcineurin, with the major and common secondary effect being cessation of IL-2 release from T-lymphocytes. However, it has to be pointed out that

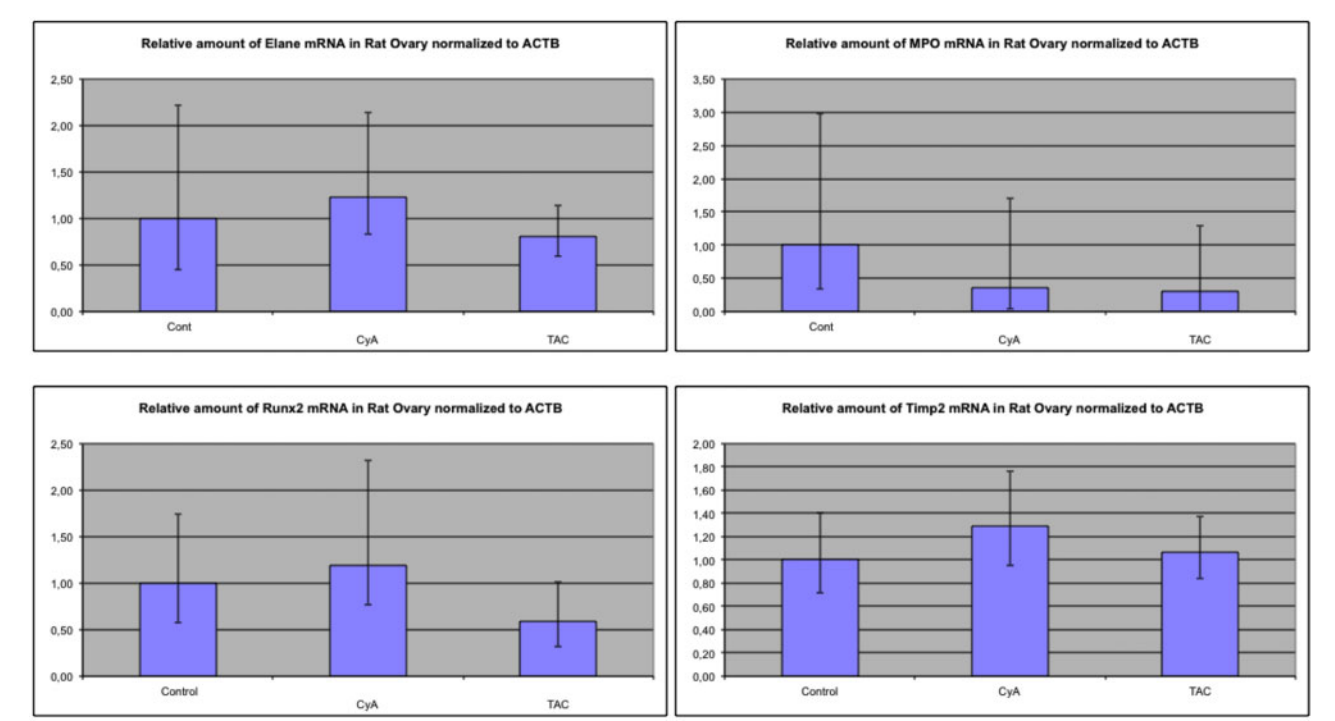

Figure 3. Analysis of ELANE, MPO, RUNX2 and TIMP3 mRNA expression levels in rat ovary. Columns represent fold changes (*y*-axis) using the control group as reference (1-fold change). Bars represent SDs of delta-Ct measurements. ACTB, actin beta; ELANE, elastase neutrophil expressed; MPO, myeloperoxidase; RUNX2, runt-related transcription factor 2; TIMP3, tissue inhibitor of metalloproteinases.

differential effects of tacrolimus and cyclosporine-A on biochemical pathways have been described in other organs and tissues such as the kidney and endothelium (Setkowicz *et al.*, 2004). A plausible explanation for such effect could be that different calcineurin inhibitors act through diverse molecular mediators: while tacrolimus binds to calcineurin through a complex with FK-binding protein family proteins, cyclosporine-A binds to calcineurin through a cyclophilin protein complex. The binding sites of FKBP and cyclophilin to calcineurin contain different amino acids, which induce distinctive conformational changes and subsequently differential activity patterns (Huai *et al.*, 2002).

It is well described that leukocytes, especially macrophages and neutrophils, are important in ovulation. Previous experiments have shown that leukocyte supplementation to the perfused rat ovary would considerably increase ovulation rate, while the depletion of neutrophils or macrophages from peripheral blood decreases it (Brännström *et al.*, 1995; Wu *et al.*, 2004). In our study, the reduction in ovulation rate found in the CyA-group might be explained by the changes in leukocyte infiltration patterns since histological examination of the post-ovulatory ovaries found a significant decrease in terms of number and distribution of lymphocytes compared to the Control group. The distribution of neutrophils, monocytes, eosinophils, and basophils did not vary between experimental groups, although there were minor differences when comparing with the Control group. These facts would indicate that calcineurin inhibitors would slightly affect vascular permeability and recruitment of immune cells to the ovary. There exist controversial data in the literature regarding the ability of calcineurin inhibitors to prevent or decrease leukocyte infiltration in different tissues: while most of the available evidence suggest that calcineurin inhibitors may affect the chemotaxis of neutrophils exerted by macrophages (Garcia-Criado *et al.*, 1997; Sasakawa *et al.*, 2000; Chang *et al.*, 2016) and also increase endothelial activation resulting in enhanced neutrophil infiltration and activation of macrophages (Thomale *et al.*, 2007) and neutrophils (Garcia-Criado *et al.*, 1997), there are also studies that have found a lack of effect of calcineurin inhibitors on neutrophil, macrophage or lymphocyte infiltration (Bayer *et al.*, 2013).

In this study, we used ELANE as a surrogate marker of neutrophilic infiltration because it has been shown that calcineurin inhibitors only alter the expression of this enzyme during hematopoiesis (Atilla *et al.*, 2001). In mature neutrophils, elastase and myeloperoxidase accumulate in intracellular granules until they undergo exocytosis during neutrophil activation (Gilman-Sachs *et al.*, 2015). The presence of neutrophils, also shown here using a surrogate marker in RT-PCR, improves the reproducibility of the findings and provides an alternative to using a histological marker. Indeed, during a parallel experiment using a set of 10 animals, neutrophil phenotyping was performed with a battery of commercially available anti-rat antibodies (data not shown). We found a very low specificity for all of them, indicated by non-specific staining of other leukocyte subpopulations, mainly macrophages. This would explain some of the discordant results that exist in the literature regarding the role of neutrophils in ovulation (Chun *et al.*, 1993; Brännström *et al.*, 1995; Oakley *et al.*, 2010).

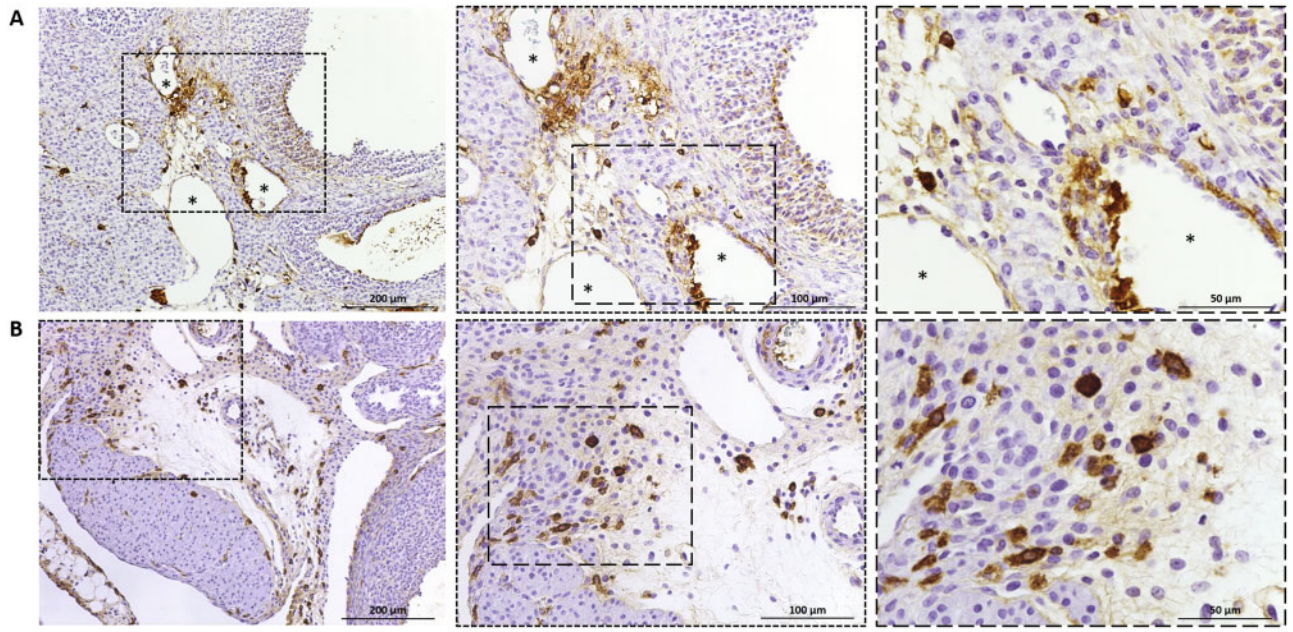

Figure 4. Macrophage distribution within the rat ovaries. Two main patterns of CD163+ cells were identified in rat ovaries. (**A**) Macrophages around the vascular structures (asterisks). (**B**) Macrophages within the newly formed corpus luteum. The rectangles delineated by dotted lines are shown at three different magnifications. These images illustrated ovaries from animals in the tacrolimus group.

The expression of MPO, like other enzymes involved in the production of free radical species, can be decreased by the effect of immunosuppressive drugs (Horl *et al.*, 1989). Consequently, we observed that MPO mRNA expression was reduced both in the TAC and CyA groups, indicating that although there is no difference in the number of neutrophils (measured by ELANE), their capacity to produce reactive oxygen species is diminished by the immunosuppressive drugs.

When we compared mRNA levels of the molecular markers of ovulation, although there were no statistically significant differences between groups, a tendency toward downregulation of RUNX2 was observed in animals treated with tacrolimus. On the other hand, TIMP3 expression was similar between groups. This is consistent with the fact that the intracellular signaling pathways involved in TIMP3 regulation seem to be independent of calcineurin (Garcia-Alvarez *et al.*, 2006). These findings suggest that the detrimental effect on ovulation rate observed in the group of animals treated with cyclosporine-A is independent of the expression of metalloproteinase inhibitors.

The main limitation of this study is that it was designed to detect differences in ovulation rates; given the complexity of the process of ovulation, this experiment allowed only a limited analysis of the mechanisms behind such an effect. We rule out that the decrease in the ovulation rate caused by cyclosporine-A is due to an abnormal infiltration of the ovary by leukocytes subpopulations, and it also seems very unlikely that imbalances in the production of metalloproteinase inhibitors could explain the observed impaired ovulation. There are other factors, such as changes in production of metalloproteinases (Atilla *et al.*, 2001), abnormal vascularization (Jiang *et al.*, 2015) or the contractility of follicular wall (Takeda *et al.*, 1993), that should be further explored.

To conclude, we have provided evidence, for the first time, that cyclosporine-A decreases ovulation rate in a rat model. The findings of this study suggest the preferred calcineurin inhibitor for organ transplanted females who are aiming for pregnancy should be tacrolimus, rather that cyclosporine-A.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

F.Z., C.D.G., and M.B. contributed equally to the design of the experiment, analysis of the data and writing of the manuscript. M.S. participated in the writing of the manuscript and analysis of the results. The animal work was done by C.D.G. in collaboration with F.Z. Immunostaining and related techniques were performed by E.N., F.Z., and C.D.G.

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Conflict of interest

There is no conflict of interest.

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