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17. Experimental Protocol

a) In this section describe your experimental protocols, outside of normal husbandry, to be performed on the animals. **This response should provide the committee with a clear understanding of what specifically happens sequentially to each animal or group of animals and over what time period.** It is not necessary to repeat the surgical description that is provided in question 28, but the timing of the surgery within the experiment should be indicated. Be sure to include: all drugs given, including dosage range, routes and frequency of administration; nutritional intervention; social or environmental manipulation; method and amount of biological samples taken; methods of antibody production; use of radioactive materials, blood or other fluid sampling including method and amount, etc. Specify the expected sequence, frequency and duration of these procedures. **If this protocol is to cover an animal colony, use this section to detail breeding procedures/methods.** (Append additional page(s) if necessary)

For *in vitro* fertilization we will collect oocytes and semen from selected SIV infected animals.

Stimulation of macaques for oocyte collection: Beginning on days 1-3 of the menstrual cycle, rhesus monkeys receive twice daily injection im of 30 IU recombinant human FSH in 0.5 ml saline (Ares Advanced Technology) for 7-9 days. Ovaries are examined on day 7 or 8 of treatment via ultrasonography while under ketamine restraint (15 mg/kg IM), if appropriate, to evaluate the follicular response to gonadotropin stimulation. If several 4-5 mm follicles are present, 1000 IU of recombinant human chorionic gonadotropin in 0.5 ml saline (hCG, Ares) is given i.m. 1 day following the last day of FSH treatment. for induction of oocyte maturation. Oocytes are aspirated laparoscopically under ketamine anesthesia 27-30 h following the injection of hCG (day 9-13 of the cycle). Animals will be rested for at least one **menstrual** cycle following stimulation

and laparoscopy before undergoing a subsequent induction. **Animals will receive a maximum of 5 stimulation cycles in a 12 month period.**

Macaque semen collection- **Males will be tranquilized with a low dose (5-10 mg/kg i.m.) of ketamine, and penile electrostimulation will be performed as previously described [Gould and Mann, 1988]. For this approach, strips of defibrillation pads will be used, rather than tin foil, to prevent any risk of penile trauma from the higher voltage required. Current (4-10 mamp) will be applied at 30 Hz for 10-15 sec at 10-sec intervals using a Grass S5 stimulator unit (Grass Medical Instruments). Electrical charges are generally delivered at a frequency of 10-20 impulses per second for 25-50 milliseconds.**

Although unlikely, in the event that penile electrostimulation is unreliable in tranquilized animals, rectal probe stimulation will be done as previously described [Wildt et al., 1986; Gould and Mann, 1988]. Animals are fully anesthetized with ketamine (15mg/kg i.m.) prior to the procedure. The electrical stimulus of 30 Hz sine wave will be applied using a rectal probe with a diameter of 1.3 cm. Electrical stimuli will be given in sets of 10 serial stimulations applied at the same voltage and amperage. The stimuli are given in 3-second on and 3-second off pattern, with a continuous rise in voltage from 0 volts to the desired peak, then returning to 0. Initial voltage is selected on the basis of the animal's response (predominantly leg extension) during stimulation. After the first 10 stimulations at initial voltage, the next set of 10 stimulations is increased 1 volt. After three sets (total of 30 stimulations), the procedure is discontinued and the ejaculate evaluated. If additional ejaculate is required, another series of 30 stimulations is administered, but the first ten stimulations are initiated at the intermediate voltage of the first series and increased by 1 volt for each additional series of 10 stimuli. No more than 8 volts will be applied, as this often results in urine contamination. **The minimum interval between electroejaculations for an individual male will be 3 days. The maximum number of electroejaculations a single male will receive in a 30 day peroiod will be 6.**

Non-surgical rhesus embryo transfer - **For this procedure, blood samples (2ml) will be collected from potential recipients daily for 5 days for determination of the LH peak. Prior to surgery , animals are deprived of food (≤16 hours) and water (≤3 hours).** Rhesus monkeys will be anesthetized and prepared for the transfer procedure as we have previously described (Wolfgang, J. Med. Prim. 30:148-155, 2001). Transfer procedures are conducted under ketamine anesthesia (15 mg/kg IM). The cervix will be cannulated and after placement of the cell sampler, sterile tubing connected to a micrometer or standard hamilton glass syringe will be inserted through the cell sampler. The tubing will have the embryo already drawn up in the distal end, and will be gently expelled in 50 μl or embryo culture less medium. Following successful delivery of the embryo, recipients may receive either progesterone implants or progesterone injections as described below. Recipient animals may be bled three times per week for three weeks, and plasma progesterone and CG will be determined by RIA. The volume of blood withdrawn will not exceed more than 6 ml/kg of body weight in a 2 week period, at which time we will assess hematocrit and administer iron supplements and/or alter sampling regimen, upon consultation with the veterinarian. Throughout pregnancy the status of the fetus will be monitored by transabdominal palpation of the uterus or ultrasound, until term. Depending on the individual, ultrasound may be done with brief physical or ketamine (15 mg/kg) restraint. **The minimum interval between ultrasound exams requiring anesthesia will be 1 month. The maximum number of ultrasound exams requiring anesthesia will be 5.**

Steroid hormone administration to rhesus monkeys - One reason for a low success rate in nonsurgical uterine embryo transfer is the asynchrony of the recipient female uterus and donor embryos. In order_to create the correct uterine environment to support pregnancy, we may administer exogenous progesterone by one of two approaches.

1. Progesterone will be delivered either by injections or by silastic implants. Silastic tubing (Medical grade silastic tubing, 0.335 cm inner diameter by 0.465cm outer diameter) cut to a 6 cm length will be packed with crystalline progesterone, and sealed at the ends with silastic adhesive. Implants are sterilized by **Gas (ethylene oxide) sterilization**, followed by storage until needed. 30 minutes prior to insertion, the implants are soaked in 3% Betadine in saline. Animals will be anesthetized with ketamine (15 mg/kg) the day after embryo transfer, and the tubing will be placed subcutaneously through a ~1 cm incision in the intrascapular area, which will then be glued shut **using surgical glue**. These implants have been shown in the literature (below) to result in sustained luteal phase progesterone levels and maintenance of a secretory endometrium, which will maximize the chances of a successful implantation and establishment of pregnancy. We will monitor progesterone and chorionic gonadotropin levels in peripheral serum samples. We may collect blood samples as often as daily

from the saphenous or femoral veins starting the day of transfer until day 40 of pregnancy. These blood samples will be calculated to remove no more than 6 ml/kg of body weight in a 2 week period, at which time we will assess hematocrit and administer iron supplements and/or alter sampling regimen, upon consultation with the veterinarian. The sampling regimen frequency may be changed to accommodate these guidelines. In animals with continuing pregnancies, we may collect blood samples of up to 10 ml as often as weekly until the week after fetectomy. Hormone levels will be measured by radioimmunoassay (RIA) to establish the levels of progesterone released, and the presence of an implantation and establishment of pregnancy. The capsules will remain in place until day 40 of pregnancy. If pregnancies appear to be compromised by removal of the capsules, we may simply leave them in place or add additional capsules as needed to achieve appropriate progesterone levels.

2. As a reliable and simple alternative delivery method, progesterone injections of 6 mg in oil will be administered one time per day in the early morning. Progesterone will be delivered by daily im injection (in 100 μl corn oil, 0.8 mg/kg). These levels are based on typical regimens in human IVF clinics. In the previous experience in the Reproduction Research Service unit, we have given injections for approximately two weeks and confirmed pregnancy by chorionic gonadotropin RIA and ultrasound to confirm maintenance of pregnancy. In the event that 2 weeks of progesterone injections are inadequate to sustain pregnancy, progesterone may be administered for up to one month, which would cover the time that the corpus luteum is required. Progesterone is given through the first trimester (11 weeks) in human IVF clinics, however 2-4 weeks will be sufficient for the rhesus monkey.

Hogden, G.D. Surrogate embryo transfer combined with estrogen-progesterone therapy in monkeys. 1983 JAMA 250, 2167-2171.

Longcope, C., Bourget, C., Meciak, P.A., Okulicz, W.C., McCracken, J.A., Hoberg, L.M., Padykula, H.A. (1988). Estrogen dynamics in the female rhesus monkey. Biol. Repro. 39, 561-565.

Okulicz, W.C. and Balsamo, M. (1993). A double immunofluorescent method for simultaneous analysis of progesterone-dependent changes in proliferation and the oestrogen receptor in endometrium of rhesus monkeys. J Repro Fert 99, 545-549.

Okulicz, W.C., Savasta, A.M., Hoberg, L.M., Longcope, C. (1990). Biochemical and immunohistochemical analyses of estrogen and progesterone receptors in the rhesus monkey uterus during the proliferative and secretory phases of artificial menstrual cycles. Fert. Sterl. 53, 913-920.

Rudolph-Owen, L.A., Slayden, O.D., Matrisian, L.M., Brenner, R.M. (1998). Matrix metalloproteinase expression in Macaca mulatta endometrium: evidence for zone-specific regulatory tissue gradients. Biol. Repro. 59, 1349-1359.

Laparoscopic oocyte collection and embryo transfer: Females will be anesthetized with ketamine (15 mg/kg IM) followed by intubation and administration of isofluorane (1.5 % in 2 liters oxygen/minute). The abdominal area will be shaved and cleaned. After insufflation with air to 15 mmHg over atmospheric pressure, a small (< 1 cm) midline incision will be made, through which the laparoscope will be introduced to visualize the ovaries. A 12 gauge trochar will be introduced through the abdomen lateral to the laparosope, and follicles will be aspirated via a needle inserted through the trochar. For embryo transfer, the ovaries are immobilized and two embryos are transferred in a small volume (< 1 ml) of sterile culture medium through sterile teflon tubing inserted approximately 3 cm into the oviduct through the fimbriated opening. After aspiration 100 ml of sterile saline is administered through the laparoscope to help prevent adhesions, and incisions will be closed with sutures. Aspiration or transfer of oocytes may be done up to 6 times per monkey, with a rest cycle between each procedure. Monkeys are evaluated by the surgeon at each laparoscopy for evidence of adhesions or endometriosis and the attending veterinarian will be consulted if there is any question of an animal's suitability in the project. No animal will have more than six laparoscopic procedures in their lifetime. A review of the surgical history of each potential recipient will be done to adhere to this limit.

Surgical transfer of macaque embryos: We are carrying out nonsurgical, transcervical embryo transfer to minimize stress on the animals and develop the most efficient, least invasive embryo transfer methods. However, this is a new approach and the efficiency of nonsurgical transfer of rhesus embryos to the uterus through the cervix may turn out to be suboptimal, or not appropriate for certain individuals because of the structure of the cervix. Surgical transfer of early or late stage embryos to the oviduct has been very successful at both the Wisconsin and Oregon Primate Research Centers (~40% of embryo transfers results in a pregnancy). **For this procedure, blood samples (2ml) will be collected from potential recipients daily for 5 days for determination of the LH peak. Prior to surgery , animals are deprived of food (≤16 hours) and water (≤3 hours).** Females will be anesthetized with ketamine (15 mg/kg IM) followed by intubation and administration of isofluorane (1.5 % in 2 liters oxygen/minute). The abdominal area will be shaved and cleaned. The oviduct is exteriorized through a midline abdominal incision, the ampulla of the oviduct is catheterized with sterile teflon tubing and the embryo is delivered into the oviduct in a small volume (<10μl) of sterile buffer. **Each animal will undergo up to 4 surgical embryo transfers.** Complete histories of all animals are reviewed before any surgical procedure is initiated. **Recipient animals may be bled three times per week for three weeks, and plasma progesterone and CG will be determined by RIA. If pregnant, recipient animals may continue to be**

bled once weekly until term. The volume of blood withdrawn will not exceed more than 6 ml/kg of body weight in a 2 week period, at which time we will assess hematocrit and administer iron supplements and/or alter sampling regimen, upon consultation with the veterinarian. Throughout pregnancy the status of the fetus will be monitored by transabdominal palpation of the uterus or ultrasound, until term.

Artificial Insemination Procedure. Female sex skin coloration is monitored daily. Starting approximately 8 days after the onset of menstruation, 2 ml blood samples are taken daily to determine LH values and supplement color data. Females are drawn until their LH levels rise or until their sex skin begins breaking down. The LH peak and color data is used to determine when an artificial insemination (AI) will take place. This is usually done on the day of the LH peak or up to 2 days after. A semen sample is collected by electroejaculation, and the semen is drawn into a sterile syringe with a 5 inch 18 gauge blunted needle attached. The female is anesthetized with ketamine(15 mg/kg IM). Her perineal area is scrubbed with betadine and rinsed with water, and she is laid ventrally on a slanted platform, head facing downward. The tail is taped up to her back to keep it away from the area for better viewing and to avoid contamination. A cell sampler (similar to the device used for cervical cannulation and embryo flushing) is inserted into the cervix and the semen delivery needle is put through the cell sampler and the semen is introduced either directly into the uterus, or at the cervical os, depending on the cervical structure in an individual animal. The animal is then allowed to recover from anesthesia in a recovery cage and placed in her home cage when she is recovered.

b) Do any animals undergo any type of restraint beyond normal housing methods?
YES If YES, indicate
method, length of restraint, and justification for such restraint. If the design of the study requires continuous restraint for longer than 12 hours without the opportunity for exercise, be sure the justification addresses need for such an extended period and include the maximum length of time the animals will be restrained. Include any plans for providing additional enrichment and any steps taken to avoid physical discomfort during the restraint. (See Campus Policy on Non-human Primate Chairing if applicable - available on the web at: www.rarc.wisc.edu

Blood draws and semen collection will be accomplished on animals chemically restrained by intramuscularly administered ketamine hydrochloride at 5-20 mg/kg dose.

c) Are any animals subjected to fluid or food restriction? **YES**

Since under general anesthesia some animals vomit, to avoid unnecessary complications on the day of blood or semen sample collection performed under anesthesia morning food and water is withdrawn. Animals will not be deprived of water longer than 3 hours prior to the procedure.

d) Will any animals require nonstandard husbandry exemption (e.g. exercise exemption, extended cage cleaning periods, etc.)

YES

For animals that are infected with immunodeficiency disease inducing viruses individual housing is the accepted practice. This practice is maintained in order to limit the spread of emerging pathogens from one animal to the other.
Sperm or oocyte donors will be selected from SIV infected animals therefore they will be single housed in special containment area of the WPRC designated to perform AIDS related biomedical research.

Embryo recipient animals from SIV-infected gamete/oocyte donors will be considered to be in a low risk group, but will be housed separately from the general colony. They will be pair housed in Center Annex (Building II) second floor, but separate from other SIV-exposed rhesus. Testing of animals for seroconversion will continue for 6 months postpartum. Animals who do not seroconvert can be used for additional embryo transfers, or will be returned to the general colony. Newborn animals will be tested for the presence of SIV for at least 6 months postpartum. SIV negative progenies will be weaned according to accepted WPRC procedures and returned to general colony. Seroconverted animals will be assigned to other so far undetermined AIDS related projects, and they remain in the WPRC AIDS research animal area.