

PROTOCOL FOR ANIMAL USE AND CAREEmail to: campusvet@ucdavis.edu**CNPRC**

EH&S USE ONLY

PROTOCOL: 10397
EXPIRES: 12/19/04

Investigator		Contact	
Last Name:		Last Name:	
First:		First:	
Middle:		Middle:	
email:		email:	
Department:		Department:	
Phone / Fax:		Phone / Fax:	
After hrs. #:		After hrs. #:	

Species (common names):	Number:	Source:
Rhesus macaque	6	CNPRC

Project Title	Blockade of VEGF Action in Macaque Uterus.		
Overnight housing location::	CNPRC	Day use only :	
Animals will be maintained by:	<input checked="" type="checkbox"/> Vivarium <input type="checkbox"/> Investigator (If investigator maintained, attach husbandry SOP's.)		

Procedures: Provide a one or two sentence layman's description of the procedures employed on the animals in this project. This information will help the animal care staff understand any conditions they may encounter while caring for your animals.

All animals will be ovariectomized prior to group placement. All animals will be treated with estradiol (E₂) and progesterone (P) implants, to induce an artificial menstrual cycle. At the end of the artificial cycle, both implants will be removed to induce menstruation. The endometrium will be collected after 8 days from the onset of menstruation, by hysterectomy. All animals will be monitored for presence of vaginal bleeding after hormone withdrawal.

Special Husbandry Requirements: Describe any special requirements your animals have with respect to **food, water, temperature, humidity, light cycles, caging type, bedding**, or any other conditions of husbandry.

No special husbandry required.

Other instructions for animal care staff: (check applicable entries)

Sick Animals	Dead Animals	Pest Control
<input checked="" type="checkbox"/> Call Investigator	<input checked="" type="checkbox"/> Call Investigator	<input type="checkbox"/> Call Investigator
<input checked="" type="checkbox"/> Clinician to treat	<input checked="" type="checkbox"/> Save for Investigator	<input checked="" type="checkbox"/> OK to use pesticides
<input type="checkbox"/> Terminate	<input type="checkbox"/> Bag for disposal	<input type="checkbox"/> No Pesticides in animal area
<input checked="" type="checkbox"/> Necropsy	<input checked="" type="checkbox"/> Necropsy	

Hazardous Materials (only if in the animal room):

Infectious Agents?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Radioisotopes?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Chemical Carcinogens?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Toxic Chemicals?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	

Funding source:	Mellon Foundation	Previously approved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No
Is the project already funded?	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	Previous protocol number (if any):	

What Veterinarian or veterinary clinic will provide care for your animals? (check one)

<input type="checkbox"/>	Lab Animal Health Clinic (2-0514)	<input checked="" type="checkbox"/>	California Primate Research Center (2-0447)
<input type="checkbox"/>	VMTH Large Animal Field Service (2-0292)	<input type="checkbox"/>	Another Veterinarian

If you checked "Another Veterinarian", please provide:

Veterinarian:		Address:	
Day phone:			
Emergency phone:		Email:	

If your veterinarian is not affiliated with one of the three service units listed above, please contact the campus veterinarian, 2-2357 (email pctillman@ucdavis.edu) for current information about training and record keeping requirements.

Summary of Procedures:

a) Briefly describe the **overall intent** of the study. Include in your description a statement of your hypothesis, the objectives and significance of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon.

In adult female primates, the endometrium undergoes shedding of the upper zones and cyclical repair and regeneration during the normal menstrual cycle. A key feature of this remarkable tissue remodeling is the growth of the vasculature. At the end of menstruation, the remaining deeper zones contain ruptured arterioles and venules. By day 5 after onset of menses, the blood vessels have healed and the surface epithelium has spread and covered the ragged surface. Subsequently there is a rapid growth of vasculature that supplies the upper layer of the regenerating endometrium.

Recently, we have reported two important findings on the pattern of vascular endothelial growth factor (VEGF) expression and endothelial cell proliferation during early- to mid- proliferative phase in the rhesus macaque endometrium: 1) After menstrual sloughing of the upper zones (5-6 days after **progesterone withdrawal**), the newly formed surface epithelium showed a dramatic increase in VEGF mRNA expression during the early-proliferative stage. Also, there was significant increase in Flt-1 and KDR mRNA expression in multiple profiles of small blood vessels just below the newly formed surface epithelium. In hormone deprived (HD) animals (after both **estradiol and progesterone withdrawal**), exactly the same pattern of up-regulation and localization of VEGF, Flt-1, and KDR mRNAs were found through HD 5-6 days in the surface epithelium and vascular endothelium, indicating that these up-regulation of VEGF and its receptors expression are **independent of estradiol action**. It may be noted that menstruation and healing of endometrium occur normally with or without the presence of estradiol after progesterone withdrawal. Several studies suggest a temporal and spatial correlation between the expression of VEGF and its receptors in cutaneous wound healing. For example, it has been shown that there is pronounced expression of VEGF in proliferating keratinocytes of the newly formed epithelium and heightened expression of Flt-1 and KDR in the capillary vessels in close vicinity to the epithelium during wound healing, which is consistent with our observations during postmenstrual healing of endometrium. *Therefore, we hypothesize that VEGF plays an important role in postmenstrual healing and repair of endometrium.* 2) Our second observation was that vascular endothelial proliferation in the endometrium showed a dramatic **estrogen-dependent peak** during the mid-proliferative phase. This proliferative peak coincided with a peak in VEGF expression in the endometrial stroma. *These results indicate that VEGF of stromal origin may play an essential role in the endometrial vascular proliferation.*

To test these hypotheses, we obtained a new compound from Regeneron Pharmaceuticals, called VEGF-Trap, which is a soluble form of the VEGF receptor that can bind to VEGF. VEGF-Trap is a very potent inhibitor of VEGF action *in vivo* and *in vitro*. We used six ovariectomized rhesus monkeys for this study (conducted at the Oregon National Primate Research Center), three controls and three treated animals (n=3). The animals were treated with VEGF-Trap at a dose rate of 12.5 mg/kg intravenously on days 2, 4, and 6, and the endometrium was collected on day 8, after progesterone withdrawal. The control animals were treated similarly with the vehicle. The results are very interesting and clearly demonstrate that VEGF-trap can block the E₂-dependent mid-proliferative peak in endometrial vascular proliferation, despite the increase in VEGF mRNA expression in the endometrial stroma after VEGF-Trap treatment. Additionally, we have noted

several indications of delay in healing of endometrium after VEGF-Trap treatment, including prolonged bleeding and focal hemorrhagic lesions on the surface epithelium. However, these effects are marginal and inconclusive, and we believe that the rapid proliferation of glands and stroma under the influence of estradiol have covered the ragged surface. We are expecting a dramatic inhibitory effect of VEGF-Trap on healing of endometrium in the absence or very low serum concentrations of estradiol which is typically seen during natural menstrual cycle, and propose to test the effect of VEGF-Trap on endometrial healing in hormone deprived animals i.e. after both estradiol and progesterone withdrawal menstruation. These findings will provide new insights on the role of VEGF on healing and regeneration of primate endometrium.

b) Procedures employed in this project:

Please check the appropriate boxes if any of these procedures will be employed in your project:

- | | | |
|---|--|--|
| <input type="checkbox"/> Monoclonal Antibody Production ** | <input type="checkbox"/> Food or water restriction | <input type="checkbox"/> Special diets; food or water treatment. |
| <input type="checkbox"/> Polyclonal Antibody Production ** | <input type="checkbox"/> Non-recovery surgical procedures | <input type="checkbox"/> Induced illness, intoxication, or disease |
| <input type="checkbox"/> LD 50 or ID50 studies. | <input checked="" type="checkbox"/> Survival surgical procedures | <input type="checkbox"/> Death as an endpoint (see i below) |
| <input checked="" type="checkbox"/> catheters, blood collection, intubation | <input type="checkbox"/> Multiple survival surgery | <input type="checkbox"/> Trapping, banding or marking wild animals |
| <input type="checkbox"/> Prolonged restraint. (8 hrs+) | <input type="checkbox"/> Behavioral modification. | <input type="checkbox"/> |
| <input checked="" type="checkbox"/> Fasting prior to a procedure. | <input type="checkbox"/> Aversive conditioning. | <input type="checkbox"/> |

** If this protocol only describes antibody production, you may use the attached antibody production page in lieu of completing section c below.

c) Describe the use of animals in your project in detail, with special reference to any of procedures checked above. Include any physical, chemical or biological agents that may be administered. List each study group, and describe all the specific procedures that will be performed on each animal in each study group. Use terminology that will be understood by individuals outside your field of expertise. *(Note: This cell will expand to whatever length you require. You may make this section as long as you wish, but try to be concise. Some projects may require one or two pages.)*

All animals will be anesthetized with ketamine (10 mg/kg), intubated and placed on isoflurane anesthesia. The animals will be given atropine (0.04mg/kg) and receive a standard ovariectomy by CNPRC surgical staff. Following surgical recovery, six ovariectomized adult rhesus macaques will be split into a treatment (Group-1) and control (Group 2) group. Macaques from both groups will be treated sequentially with estradiol (E₂) and progesterone (P) implants in order to create artificial menstrual cycles. All animals will receive the E2 implant (a 3 centimeter (cm) Silastic capsule containing E2) which will be inserted subcutaneously (s.c.) in the suprascapular region while animals are briefly anesthetized. After 14 days (study day 14), all animals will receive the P implant (a 6-cm Silastic capsule containing P), inserted in the same area and manner described above. Both implants will remain in place for 14 additional days (study day 28). On study day 28, both the E₂ and P implants will be removed through a 1 cm cutaneous (skin) incision while animals are briefly anesthetized. The removal of the capsules will be done by gently palpating area for presence of capsule, making a small incision where end of capsule is detected. Using surgical instruments such as forceps, the capsule will be removed. After removal, area will be sutured and monitored daily for healing. All animals will be monitored for the presence of vaginal bleeding by vaginal swabbing after implants are removed (study day 28 through 36).

The treatment group animals (group 1) will be treated with VEGF-trap at a dose rate of 12.5 mg/kg body weight. The test article will be diluted in 10ml of phosphate buffered salt solution (PBS)) and administered intravenously on days 2, 4, and 6, after onset of menstruation (study days 30, 32, and 34). On study day 36 the animals will undergo a hysterectomy while under general anesthesia. The control animals (group 2) will be treated similarly with the vehicle (PBS) using the same schedule as outlined for the treatment group. In addition, these animals will also undergo a hysterectomy as described above. Prior to surgery, animals in both groups will receive an intravenous infusion of 10 ml/ animal/ timepoint of Br-dU (5-bromo-2'-deoxyUridine @ 10mg/ml of PBS) at three time points (24, 16, and 2) hours before hysterectomy. Br-dU is a thymidine analog selectively incorporated into cellular DNA during S-phase. This will be used to identify proliferating cells (cells making new DNA) at the end of the experiment using immunocytochemistry.

All animals will have blood collected (5 cc/timepoint) from cephalic vein using arm-pull technique, with 5cc syringe, 22 ga. needle. Blood will be collected on study days 1 (1 day after e2 implant), 15 (1 day

after P implant), 29 (1 day after implants are removed) and 36 (the day of surgery).

All animals will be returned the CNPRC colony at the conclusion of the experiment.

Note: (i) VEGF-Trap is a very potent inhibitor of VEGF action, and systemic administration of this compound at very high doses (25 mg/kg/d, sc) has shown no signs of toxic effects in several species including marmoset, rhesus, stump-tail, and cynomolgus monkeys.

(ii) Br-dU is not a radioisotope. At the Oregon National Primate Research Center, we () and others have used the above doses of Br-dU in rhesus monkeys without any toxic effects.

d) Study Groups and Numbers: Define, in the form of a table, the numbers of animals to be used in each experimental group described above. The table may be presented on a separate page as an attachment to this protocol if you prefer. The Normal format should be three columns: Study Group, Procedure, Number of animals. The number of rows should follow from the number of study groups; **you may add as many rows as you require**. The chart must fully account for the number of animals you intend to use under this protocol. Assign each group to an invasiveness category according to the chart below.

Group	Procedures / Drugs	Number of Animals	Category
1	Intravenous VEGF-trap infusion	3	3
2	Intravenous vehicle (PBS) infusion	3	3

Categories of invasiveness

Category	Description
1	Little or no discomfort or stress Examples: domestic flocks or herds being maintained in simulated or actual commercial production management systems; the short-term and skillful restraint of animals for purposes of observation or physical examination; blood sampling; injection of material in amounts that will not cause adverse reactions by the following routes: intravenous, subcutaneous, intramuscular, intraperitoneal, or oral.
2	Minor stress or pain of short duration Examples: cannulation or catheterization of blood vessels or body cavities under anesthesia; minor surgical procedures under anesthesia, such as biopsies or laparoscopy; short periods of restraint beyond that required for simple observation or examination, but consistent with minimal distress
3	Moderate to severe distress Examples: major surgical procedures conducted under general anesthesia, with subsequent recovery; prolonged (several hours or more) periods of physical restraint; induction of behavioral stresses such as maternal deprivation
4	Severe pain near, at or above the pain tolerance threshold Examples: exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs, chemicals, or infectious agents at levels that markedly impair physiological systems and which cause death, severe pain, or extreme distress; Surgical experiments which have a high degree of invasiveness.

Further descriptions of these categories are included in the instructions following this document.

e) **Rationale for species and numbers:** How did you determine that 1) the species choice was appropriate and 2) the number of animals in each study groups was the minimum number necessary to achieve sound scientific results?

The aim of this study is to gain new knowledge regarding mechanisms of endometrial vascular development and healing after menstruation in primates. Rhesus macaques are essential for this work because they menstruate identically to women. Endometrial physiology including regulation by steroid hormones and menstruation, have been studied more intensively in rhesus macaques than any other nonhuman primate species. The rhesus macaque endometrium differentiates into morphological zones that are very similar to those of women. Moreover, we would like to compare the results of this study with our previous findings in the rhesus macaque model.

This is not a pilot study. The procedures and the number of animals (n=3) proposed in this study are the minimum number of animals required for statistical validity of the results.

f) **Surgery:** If the project involves survival surgery, where will the surgery be conducted?

Building: Room:

Who will be the surgeon?

g) **Anesthetics, Analgesics, Tranquilizers, Neuromuscular blocking agents:**

Post procedural analgesics should be given whenever there is possibility of pain or discomfort that is more than slight or momentary. If postoperative analgesics are not to be given, justify the practice under part (i) below.

Provide the following information about any of these drugs that you intend to use in this project.

Species	Drug	Dose (mg/kg)	Route	When and how often will it be given?
Rhesus	Ketamine	10	IM	Once/implant insertion and removal and Ovariectomy and Hysterectomy
	Atropine	0.04	IM	Once/Surgery (Ovariectomy and Hysterectomy)
	Isoflurane	Inhaled	To effect	Once/Surgery (Ovariectomy and Hysterectomy)
	Oxymorphone	.75	IM	3X/day for 2-3 days post surgery (Ovariectomy and Hysterectomy)

h) **Neuromuscular blocking agents** can conceal inadequate anesthesia and therefore require special justification. If you are using a neuromuscular blocking agent, please complete the following:

Why do you need to use a neuromuscular blocking agent?

What physiologic parameters are monitored during the procedure to assess adequacy of anesthesia?

Under what circumstances will incremental doses of anesthetics-analgesics be administered?

i) Adverse effects:

Describe any potential adverse effects of the experiment on the animals (such as pain, discomfort; reduced growth, fever, anemia, neurological deficits; behavioral abnormalities or other clinical symptoms of acute or chronic distress or nutritional deficiency)

We do not anticipate any potential adverse effects of the experiment except minor stress or pain after hysterectomy.

How will the signs listed above be ameliorated or alleviated? If signs are not to be alleviated or ameliorated by means of post-operative analgesics or other means, explain why this is necessary.

Postoperative analgesics will be administered at the request and guidance of the veterinarian.

Note: if any unanticipated adverse effects not described above do occur during the course of the study, a complete description of those effects and the steps taken to mitigate them must be submitted to the committee as an amendment to this protocol.

Is death an endpoint in your experimental procedure? [] Yes [X] No

(Note: "Death as an endpoint" refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation). If death is an endpoint, explain why it is not possible to euthanize the animals at an earlier point in the study. If you can euthanize the animals at an earlier point, describe the clinical signs which will dictate that an animal will be euthanized.

j) Literature search for alternatives and unnecessary duplication:

*Federal law specifically requires this section. You are required to conduct a literature search to determine that either 1) there are no alternative methodologies by which to conduct this class/lab, or 2) there are alternative methodologies, but these are not appropriate for your particular class/lab. "Alternative methodologies" refers to reduction, replacement, and refinement (the three R's) of animal use, not just animal replacement. You must also show that this use of animals is not **unnecessarily** duplicative of other studies.*

UC Davis provides on-line access to a number of databases that can be used to search for alternatives. Visit

http://trc.ucdavis.edu/jawelsh/Databases/Databases_Med_Vet_Researchers.htm (email: jawelsh@ucdavis.edu)

or http://www.vetmed.ucdavis.edu/Animal_Alternatives/main.htm (email: mwood@ucdavis.edu)

What was the date on which you conducted this search?

10/29/02

List the databases searched or other sources consulted (there should be more than one). Include the years covered by the search.

Database Name	Years Covered	Keywords / Search Strategy
PubMed	1974-2002	Endometrium, menstruation, nonhuman primate, healing, progesterone withdrawal, rhesus macaque, laparoscopy, laparotomy, hysterectomy, endometrial cell culture, endometriectomy, endometrial bleeding, VEGF antagonists, VEGF-trap, etc.
Entrez	1974-2002	same
ISI Web of Knowledge	1990 to 2002	

What were your findings with respect to alternative methodologies?

The literature searches described above provided evidence that abnormal uterine bleeding is a significant cause of suffering for women which accounts for more than half of the clinical practice in gynaecology. The study further confirmed that studies of endometrial vascular development and bleeding can only be done in an in vivo model. Steroid hormones are responsible for normal and abnormal endometrial bleeding, but the identity of locally operative factors in endometrial bleeding and healing are not known. Breakthrough bleeding is the most important cause for the discontinuation of extremely effective steroid contraceptives. Systemic or local administration of VEGF antagonists can block vascular development and wound healing. Menstruation only occurs in women and nonhuman primates. Angiogenesis in the endometrium and menstruation occur as a result of complex interaction of several cell types in the primate endometrium, even by recruiting immune cells from systemic circulation. There is also a zonal gradient in expression of different tissue factors in the endometrium from cells in the functionalis to basalis. Therefore, nonhuman primates are the only suitable experimental animal model for this work and no alternatives to the proposed research exist. There are no less painful and/or stressful alternatives methods for tissue collection, and for insertion and removal of silastic implants of estradiol and progesterone.

Has this study been previously conducted?

Yes No

If the study has been conducted previously, explain why it is scientifically necessary to replicate the experiment.

k) **Disposition of animals:** At what point in the study, if any, will the animals be euthanized?

Euthanasia is not part of the experimental design, but will be at the discretion of a senior veterinarian.

l) **Methods of euthanasia:** Even if your study does not involve killing the animals, you should show a method that you would use in the event of unanticipated injury or illness. If anesthetic overdose is the method, show the agent, dose, and route.

Species	Method	Drug	Dose (mg/kg)	route
Rhesus	Overdose	Pentobarbital	60	IM

m) **Surplus animals:** What will you do with any animals not euthanized at the conclusion of the project?

Return to the CRPRC colony.

Assurances for the Humane Care and Use of Vertebrate Animals:

Principal Investigator's Statement:

I have read and agree to abide by the *UC Davis Policy and Procedure Manual* section 290-30 (Animal Use and Care). This project will be conducted in accordance with the *ILAR Guide for the Care and Use of Laboratory Animals*, and the *UC Davis Animal Welfare Assurance* on file with the US Public Health Service. (These documents are available from the Campus Veterinarian and at <http://ehs.ucdavis.edu/>). I will abide by all Federal, state and local laws and regulations dealing with the use of animals in research.

I will advise the Animal Use and Care Administrative Advisory Committee in writing of any significant changes in the procedures or personnel involved in this project.

_____ <i>Principal Investigator</i>	_____ <i>Rank / Title</i>	_____ <i>Date</i>
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Committee Use Only Below

** Conditions necessary for Committee Approval:
Final Disposition of this protocol: <input type="checkbox"/> Approved <input type="checkbox"/> Not Approved <input type="checkbox"/> Withdrawn by Investigator Date of Action: ____/____/____

I verify that the Institutional Animal Care and Use Committee of the University of California, Davis, acted on this protocol as shown above.

_____ <i>Campus Veterinarian</i>	_____ <i>Date</i>
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