

PROTOCOL FOR ANIMAL USE AND CARE*Handwritten forms are not accepted***CNPRC**

EH&S USE ONLY

PROTOCOL # 10518
EXPIRES: 3/13/04**Investigator**

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

Contact

Last Name:	
First:	
Middle:	
email:	
Department:	
Phone:	
After hrs. #:	

Species (common names):	Number:	Source:
Rhesus males	20/year	CNPRC
Rhesus females	10 /year	CNPRC

Project Title	Effects of tobacco smoke on primate sperm function and genetics		
Overnight housing location::	CNPRC	Day use only :	
Animals will be maintained by:	<input checked="" type="checkbox"/> Vivarium <input type="checkbox"/> Investigator <i>(If investigator maintained, attach husbandry SOP's.)</i>		

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.

Male monkeys will be exposed to environmental tobacco smoke in exposure chambers located at the CNPRC for 6 hours per day/5 days per week for up to 270 consecutive days. Both smoke exposed (10) and control (10) males will have semen collected up to twice weekly. Female monkeys will be treated with hormones and follicles aspirated.

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.

None other than smoke-exposed males will be housed in special exposure chambers at CNPRC

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

Sick Animals	Dead Animals	Pest Control
<input type="checkbox"/> Call Investigator	<input type="checkbox"/> Call Investigator	<input checked="" type="checkbox"/> Call Investigator
<input checked="" type="checkbox"/> Clinician to treat	<input type="checkbox"/> Save for Investigator	<input 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 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:	Phillip Morris Ext Res Prog	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):	7780

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 ptillman@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.

The deleterious effects of smoking on sperm motility and overall fertility have been observed for many years in men. However, more recent reports have shown an association of smoking with DNA damage, chromatin damage and aneuploidy in human sperm. Most recently there has been a report that smoking is associated with an alteration in the male:female ratio of offspring born to smoking parents, even if only the father smokes. However, the effects of environmental tobacco smoke (ETS) on male reproduction have not been documented and, because of confounding factors, these effects are difficult to study in humans.

We propose to use both in vitro and in vivo models of tobacco smoke exposure in the rhesus monkey to characterize the genetic effects of ETS exposure in males. The effects of ETS in vitro on the biological function, chromatin integrity, ratio of x:y sperm and rate of chromosomal abnormalities in monkey sperm will be determined. We will also evaluate the effect of ETS exposure of sperm on the subsequent development and genetic stability of developing embryos.

We will determine the effect of in vivo tobacco smoke exposure on sperm function and early embryo development for the same endpoints that will be used for the in vitro ETS exposure experiments and whether these effects are reversible after cessation of ETS exposure. By evaluating the effects of smoke exposure on sperm in vitro and in male monkeys in vivo, we can compare the relative effects of smoke on sperm and the resulting embryos after in vitro fertilization. This will help to differentiate between the effects of smoke that occur during development of sperm and those that may be the result of toxic components being transported by sperm to oocytes and resulting embryos. The monkey model will also provide an opportunity to determine effects that are solely the result of male exposure because oocytes will be obtained from females that have no smoke exposure. This project is a unique opportunity to perform controlled studies on the effects of ETS exposure on male reproduction in primates, an area in which no studies have been published in either humans or animal models.

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 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.)

Experimental Groups:

Males, Control (N= 10)

Males, in vivo smoke exposure (N= 10)

Females, oocyte donors (not an experimental treatment) (N=10)

In vivo smoke exposure: Adult male rhesus monkeys (10 per year) will be housed in standard cages within special exposure chambers located at CNPRC in the exposure facility. Animals will be exposed to aged and diluted side stream cigarette smoke for 6 hours per day, 5 days per week, for up to 270 consecutive days. For the remaining days (2 per week), they will be housed in the same location without exposure to smoke. This is a standard protocol used by other investigators at the CNPRC. The control animals will be housed in regular indoor CNPRC housing.

Semen Collection (N=20): Both the control and smoke-exposed males will have semen collected weekly using established protocols. All males maintained for semen collection wear a light-weight metal alloy collar to facilitate moving the monkey to the primate chair restraint. All animals will have received prior training for chair restraint using established CNPRC procedures. Semen will be collected from chair-trained adult male rhesus macaques by penile cuff electroejaculation a maximum of 2 times per week (Sarason et al., 1990). This procedure will include 20 male rhesus monkeys per year.

Superovulation and follicle aspiration: Female rhesus monkeys will be used to provide eggs for the experiments to evaluate sperm function and subsequent embryo development. Adult female rhesus macaques (10 per year) are given twice daily IM injections of recombinant human follicle stimulating hormone (rhFSH, 37.5 IU/day, Organon, or macaque FSH in the same quantity) for seven days. On day 7, for control experiments in which oocytes are matured in vivo, monkeys are given an IM injection of recombinant human chorionic gonadotropin (rec hCG; 1,000 IU; Serono or macaque CG in the same quantity) to simulate the natural mid-cycle LH surge and to promote the final maturation of the follicle. The monkeys are fasted the night before the aspiration procedure and anesthetized with ketamine the following morning. After delivery to ultrasound, the monkeys are prepared for the sterile aspiration procedure using established ultrasound-guided techniques (, 1990) using transabdominal access, the right and left ovaries are aspirated and the eggs removed as per published protocol. This procedure is comparable to that performed for women at infertility clinics. At the end of the procedure, the monkeys are returned to their home cage and observed periodically until the effects of anesthesia have worn off. The procedure takes less than 20 minutes from delivery of the animal to the return to the home cage, and to date, we have observed no adverse effects of this procedure on the monkeys (in 11 years we have performed more than 300 aspirations). It has been shown in our previous studies that the monkeys will develop antibodies to the human hormones and cannot be hormonally stimulated more than 5 or 6 times (average is about 3 times). All animals are given one full menstrual cycle before another stimulation cycle is attempted. In cycles following the menstrual cycle in which this procedure is performed, monkeys have conceived and the pregnancy has gone to term with normal infants, further indicating that no adverse effects are caused by these procedures.

Blood sampling. All male monkeys will have a blood sample (3cc, arm vein) when they are restrained in chairs for semen collection to monitor levels of nicotine and other cigarette smoke components. Blood will be sampled weekly during the time of smoke exposure. The total volume collected per kg body weight will not exceed CNPRC Guidelines.

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	Males, Control	10	2
2	Males, in vivo smoke exposed	10	2
3	Females, oocyte donors, NOT smoke exposed	10	2

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?

Because this project will evaluate the effects of tobacco smoke exposure on sperm function, including fertilization and subsequent early embryo development, a primate model must be used if the results are to be directly applicable to humans. Human and non-human primates share many aspects of reproduction and early embryo development that is significantly different in primates than in most other species, such as rats and mice.

Males for in vivo smoke exposure: Our extensive past experience with sperm function in rhesus macaques has shown that at least 6 males per group ARE usually required to show significant differences between treatment groups when treatments are performed in vitro and each animal is serving as its own control. Because we anticipate even more variation between treatments when different animals are serving as controls and treated, we have increased the minimum number to 8 per group. Because the exposure studies are relatively long (270 days), we feel it is essential to have 2 additional animals in case an animal becomes ill or injured by circumstances unrelated to treatment. Although both of these circumstances are very unlikely, it would not be possible to replace animals on the protocol once treatment begins because these studies will also correlate with the September to May breeding cycle of macaques. Data from all animals will be used if no animals are dropped from the protocol.

Superovulation and follicle aspiration: These studies require rhesus monkey eggs to determine the effects of tobacco smoke on sperm function and subsequent embryo development. The experiments will require approximately 600 eggs per year. Although we can use each female more than once, we now know from experience that we average approximately 3 or 4 cycles per female and obtain approximately 20 eggs per cycle. Therefore, we will require 10 females.

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?
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rhesus	Ketamine hydrochloride	10 mg/kg	IM	Once per menstrual cycle.

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)

None are anticipated based on our extensive experience.

Tobacco smoke exposure: No pain or discomfort FOR the animals is anticipated. The animals will be exposed only to sidestream cigarette smoke under conditions that would be equivalent to being in a very smokey room. We have noted in earlier experiments conducted on female rhesus monkeys by at CNPRC, a slight reduction in body weight (5%) compared with controls, but all animals appear healthy. Animals will be provided food and water ad libitum during the course of the study. A full-time technician (not animals care staff), trained in the operation of the smoking machine, is present during the 6 hours per day that the smoke exposure is performed.

Superovulation and follicle aspiration: Because follicle aspiration is an ultrasound-guided procedure, it is relatively non-invasive, and because we plan to rest each animal for one menstrual cycle between treatment cycles, there should be no complications as a result. There have been clinical reports of women undergoing up to 10 cycles of superovulation and ultrasound-guided follicle aspiration without ill effects. We now have 2 years of experience with animals receiving repeated stimulation protocols and have seen no adverse effects. We have monitored the animals which have had multiple follicular aspirations and they are able to have normal term pregnancies following the procedures (2001).

Semen Collection: This method has been used routinely at CNPRC for many years with good results. The males are not anesthetized during semen collection, but it has been reported that human volunteers did not find the penile cuff method of electroejaculation to be painful. Additionally, after the macaques are trained to the procedure, they cooperate fully during the transfer from cage to chair restraint. We have improved the procedure by using EEG gel material, instead of metal, for the electrode material. The gel-electrode material has eliminated the risk of tissue injury of the penis and we have had no lesions in over 12 years that semen has been collected by this method.

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.

Although we do not anticipate any adverse effects based on our previous experience, analgesics would be administered to alleviate any potential pain or discomfort. Analgesics will be administered at the discretion of the CNPRC veterinary staff in consultation with the investigator.

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:

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

What was the date on which you conducted this search? 6/02 & 1/03

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
Medline	1980-2002	Tobacco smoke and/or environmental tobacco smoke, primate semen, male reproduction, embryo development, fertilization
CRISP at NIH	current	same as above to determine if any similar grants had been recently funded whose work might not yet have appeared in the literature.
Pub Med	1980-2002	same as above to determine if any similar grants had been recently funded whose work might not yet have appeared in the literature.
	all, especially pre 1980	found older papers cited in more current articles

What were your findings with respect to alternative methodologies?

This grant was awarded to specifically study the in vivo effects of tobacco smoke on males with respect to sperm function and genetics. We are including some in vitro exposure studies, but in vitro exposure methods are inherently different than in vivo studies. During in vivo exposure, metabolites are formed in the body and sperm are exposed only to compounds as they exist once absorbed and metabolized. In vitro exposure also exposes sperm to particulate matter, but not to similar metabolites, therefore in vivo studies must be performed.

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?

No animals will be euthanized. All animals are returned to the colony.

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	sodium pentobarbital	60 mg/kg	IV

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

All animals will be returned to the colony.

