The Effects of Sidestream Smoke on the Rat Middle Ear

Experimental Protocol TOX-35

I. OBJECTIVE

The primary objective of this study is to evaluate the effects of sidestream smoke or low humidity on the middle ear of rats exposed to sidestream smoke (SSS). In particular, the ear of the rat will be examined for serous effusion (fluid behind the eardrum) potentially induced by exposure to sidestream smoke or low humidity. A second objective is to evaluate the effect of SSS on the rate of clearance or resolution of an experimentally induced middle ear effusion.

II. INTRODUCTION

Controversy exists in the literature regarding the contribution of parental smoking in the development of acute otitis media in children (Refs. 1-4). There have been numerous recent reports in the literature linking parental smoking with middle ear effusions and acute otitis media in children. The majority of these studies were composed of data collected from questionnaires mailed to parents regarding home environmental factors. The number of middle ear effusions was then correlated with parental smoking.

No basic science research using animal models to test the effects of SSS on middle ear effusions have been reported. In addition, no animals studies have been performed to test the effects of low humidity on production of middle ear effusions. This study is designed to test whether SSS exposure induces a serous effusion in the rat middle ear, or influences the rate of clearance or resolution of an established effusion. The potential effect of an environmental factor of low humidity on induction of serous effusions will also be examined.

III. FACILITIES AND ADMINISTRATION

Facilities

R.J. Reynolds Tobacco Company
Building 630-2
Winston-Salem, NC 27102

Contractors

Histopathology: Veritas Laboratories, Burlington, NC
Animal Care: Program Resources Inc., Winston-Salem, NC
Serology:

Study Administration

Study Director: Paul H. Ayres, Ph.D., D.A.B.T.

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R.J.Reynolds Research and Development, Inhalation Division

Principal Investigator: Hugh Lovejoy, M.D. ,Bowman Gray / Baptist Hospital Medical Center, ENT Department

Inhalation Study Technicians: Jim Corn and Delma France

IV. OVERVIEW

This study will be composed of five groups of male rats with a total of 89 animals allocated to the study. All study groups will be housed in whole body exposure chambers during the exposure phase of this study. Two groups of rats, 23 rats per group, will be exposed to SSS in nose only exposure tubes at concentrations of 0.1 mg wet total particulate matter (WTPM) per cubic meter of air or 1 mg WTPM per cubic meter of air at 40-60 % RH for 6 hours per day for 5 consecutive days. A third group of rats, 23 in the group, exposed in nose only exposure tubes to air at 40 - 60 % relative humidity (% RH) for 6 hours a day for five consecutive days will serve as sham controls. The portion of the day in which the animals will not be in nose only exposure tubes, they will be housed in the whole body exposure chambers and exposed to purified air at 40-60% RH. One ear in each of the animals in the SSS and sham exposure groups will have a serous effusion (fluid behind the ear drum) experimentally induced by the use of cold air directed into the external auditory canal prior to beginning the exposure phase (Goldie and Hellstrom, 1986). Since each ear is an anatomically distinct organ system, each ear will be considered as an independent experimental unit. These groups will be used to evaluate 1) the effect of SSS on the clearance or resolution of an experimentally induced middle ear effusion in the treated ear; and 2) the effect of SSS on the induction of a middle ear effusion in the untreated ear.

The fourth and fifth groups, 10 rats in each group (a total of 20 independent experimental units in each group), will be exposed to low humidity (0-10 % RH) or normal humidity (40-60 %RH) air, respectively, for 24 hours a day during the exposure phase of this study. These groups will not be exposed in nose only exposure tubes. These groups will be used to evaluate the effect of low humidity on the induction of middle ear effusions.

Endpoints determined during the exposure phase of the study, pertaining to the status of the middle ear, will be presence or absence of serous effusions as assessed by visual examination of the ear and tympanometry. Additional endpoints determined at the termination of the exposure phase of the study will include quality of effusion (thin vs. thick, etc.) and histopathology of the tympanic membrane, middle ear mucosa, and eustachian tube. Scanning electron microscopy (SEM) will be used to evaluate the eustachian tube on three of the rats in groups 1,2, and 3.

The experimental design is detailed in section VI. below.

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V. RECORDS TO BE MAINTAINED

Records of animal health/serology, allocation to experimental groups, animal identification and housing, animal room and chamber environment, daily exposure and equipment calibration, body weights, experimental procedures and observations, moribundity/mortality reports, necropsy findings, histopathology results, and statistical evaluation that would be required to reconstruct the experiment will be maintained. The study will be subject to periodic inspections and the final report will be reviewed by the Program Resources Inc. Quality Assurance Unit. Original data and any samples from this study will be stored at Bowman Gray Technical Center and will be the property of R.J. Reynolds Tobacco Company.

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VI. EXPERIMENTAL DESIGN

Group 1 **Sham Exposure**

Exposure: Sham exposure 6 h/day, 40-60 %RH
Nose only exposure tubes: yes, 6 h/day
Ear treatment: Unilateral induction of middle ear effusion
Number of animals: 23
Number of animals for histopathology: 20 **Animal ID #'s:** 101-120
Number of animals for SEM: 3 **Animal ID #'s:** 121-123

Group 2 **Sidestream smoke 0.1 mg/m^3**

Exposure: SSS 0.1 mg/m^3 6 h/day, 40-60 %RH
Nose only exposure tubes: yes, 6 h/day
Ear treatment: Unilateral induction of middle ear effusion
Number of animals: 23
Number of animals for histopathology: 20 **Animal ID #'s:** 201-220
Number of animals for SEM: 3 **Animal ID #'s:** 221-223

Group 3 **Sidestream smoke 1.0 mg/m^3**

Exposure: SSS 1.0 mg/m^3 6 h/day, 40-60 %RH
Nose only exposure tubes: yes, 6 h/day
Ear treatment: Unilateral induction of middle ear effusion
Number of animals: 23
Number of animals for histopathology: 20 **Animal ID #'s:** 301-320
Number of animals for SEM: 3 **Animal ID #'s:** 321-323

Group 4 **Low humidity air**

Exposure: Low humidity air 24 h/day, 0-10 %RH
Nose only exposure tubes: no
Ear treatment: None
Number of animals: 10
Number of animals for histopathology: 10 **Animal ID #'s:** 401-410
Number of animals for SEM: none

Group 5 **Normal humidity air**

Exposure: Normal humidity air 24 h/day, 40-60 %RH
Nose only exposure tubes: no
Ear treatment: None
Number of animals: 10
Number of animals for histopathology: 10 **Animal ID #'s:** 501-510
Number of animals for SEM: none

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Groups 1,2, and 3:

Rats in these groups will receive cold air treatment in the left ear once each day for 20 minutes for 3 days prior to beginning the exposure phase of the study. This cold air treatment will experimentally induce a middle ear effusion in the treated ear prior to starting smoke exposures.

The treated ear will be examined visually and by tympanometry to determine if SSS exposure alters the rate of clearance or resolution of the experimentally induced middle ear effusion when compared to the sham exposure. The untreated ear will be examined in the same manner to determine if SSS exposure induces middle ear effusion in the untreated ear.

Rats in groups 1,2, and 3 will be placed in nose only exposure tubes 0-30 minutes prior to beginning the 6 hour exposure and removed from nose only exposure tubes within 30 minutes after exposure. Rats in groups 2 and 3 will be placed in nose only exposure tubes to minimize ingestion of SSS aerosol particulate matter by preening and to reduce dermal exposure to SSS. Rats in group 1 will be placed in nose only exposure tubes to provide, an appropriate experimental control.

Groups 4 and 5:

Rats in groups 4 and 5 will not be placed in nose only exposure tubes nor will they be treated with cold air to induce a middle ear effusion. Group 4 rats will be housed in a whole body chamber in which the relative humidity is maintained at 0-10 %RH 24 hours/day during the exposure phase of the study except during weighing, ear examination, and animal husbandry activities. Group 5 rats will be housed in a whole body chamber maintained at 40-60 %RH 24 hours/day with the same exceptions as noted for group 4. The ears of rats in groups 4 and 5 will be examined and compared to determine if low humidity induces middle ear effusions in rats. This study will not examine the effect of low humidity on the rate of clearance or resolution of an experimentally induced middle ear effusion.

Scanning electron microscopy:

Three rats from groups 1,2, and 3 will be used for scanning electron microscopic examination of the eustachian tube epithelium of both ears from each animal.

VII. EXPERIMENTAL USE OF ANIMALS

A. Justification for Use of Animals:

Questionable data from epidemiology studies suggests that sidestream smoke induces middle ear effusions in children (Refs. 1-4). No experimental research has been conducted to evaluate the association of sidestream smoke exposure and induction of middle ear effusion. An experimental model of middle ear effusion has been developed in Sprague-Dawley rats by Goldie and Hellstrom,

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1986). The animal model suggests that the rat is the experimental animal of choice to objectively evaluate the association of sidestream smoke exposure and middle ear effusion. The juvenile Sprague-Dawley rat (Crl:CD/BR,VAF/Plus) was chosen for this study because it has been shown to be responsive to experimental induction of middle ear effusion and it will allow comparison to other studies conducted at this facility using this species and strain. In addition, the middle ear anatomy of the Sprague-Dawley rat is analagous to that of the human ear. 120 Male Sprague-Dawley rats 5-6 weeks of age will be ordered from Charles River Raleigh (Raleigh, NC) to provide the required number for testing. 89 Rats will be allocated to the study. Extra rats that remain after allocation into experimental groups or any other rats that are shipped in excess of 120 by the vendor will be used by the study director in methods development studies approved by the animal care committee. The number of rats in each group was selected to provide the appropriate statistical power after consultation with Dr. Tim Morgan, a statistician at Wake Forest University Medical Center.

B. Quarantine and Serological Evaluation:

Upon delivery, animals will be housed individually in transparent cages in room #47 for 14 days prior to the first exposure. These animals will be assigned a pre-study identification number which will be indicated on a cage card. This 14 day period will serve as a quarantine period with limited personnel access. Within 2 days of delivery, 5 rats will be randomly selected by staff from the Animal Biology Division for serological evaluation of antibodies to disease. The rats will be euthanized with 70 % carbon dioxide and serum collected. The serum will be tested for the following antibodies to disease: Reovirus Type 3, cilia associated respiratory bacillus, Kilham's rat virus, lymphocytic choriomeningitis virus, and Mycoplasma pulmonis. Lungs from these 5 rats will be collected and examined histopathologically to ascertain health status.

The start of the exposure phase of the study is dependent upon negative serology data being obtained on the pre-study samples, and upon a histopathology statement on the animals killed at delivery, releasing the animals from the 14 day quarantine.

C. Group Allocations:

Within 7-10 days of delivery, rats will be allocated into experimental groups such that the body weights in the groups are as homogeneous as possible. Surplus animals from the above procedure will be used by the Study Director for methods development projects approved by the animal care committee.

D. Animal Identification:

After allocation into experimental groups, animals will be

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tail-tattooed (Animal Identification and Marking Systems, Piscataway, NJ) with their permanent identification number by personnel trained by the Animal Identification and Marking Systems company. The animals will be returned to cages with cards attached recording the study number, animal number, sex, pre-study number, and study director. The following animal identification numbers will be used: Group 1, numbers 101-123; Group 2, numbers 201-223; Group 3, 301-323; Group 4, 401-410; and Group 5, numbers 501-510.

E. Animal Husbandry:

The animals will be housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments (Public Law 91-579), as set forth in CFR Title 9, Part 3, Subpart E, "Specifications for the humane handling, care, treatment, and transportation of warm-blooded animals other than dogs, cats, rabbits, hamsters, guinea pigs, and non-human primates."

During the quarantine period, the animals will be housed in room # 47 in building 630-2. The room will have controlled lighting (12 hours of darkness, from 6:00 pm), temperature (20-24 C), and humidity (40-60 %RH). Seven day continuous recordings will be kept of %RH and temperature. After the quarantine period, the animals allocated into experimental groups will be housed in individual stainless steel wire mesh cages in Hazelton 2 cubic meter whole body inhalation exposure chambers (Moss et al.,1982).

Rats housed in whole body exposure chambers will have ad libitum access to feed and water except during inhalation exposures for groups 1,2, and 3. Feed will be removed from the chamber in the moring prior to inhalation exposures for groups 1,2, and 3 and returned to the chambers after the completion of inhalation exposure. Paper lining the excreta collection pans in the whole body exposure chambers for all groups will be changed once daily in the morning as animals are prepared for inhalation exposure. This preparation involves an A.M. viability check for all groups and loading rats into exposure tubes for groups 1,2, and 3. This pre-exposure preparation will be conducted by the Animal Care staff. At the end of daily inhalation exposures, the rats will be removed from the nose only exposure tubes (groups 1,2, and 3) by the inhalation technical staff. Feed will be returned to the animals in groups 1,2, and 3 after completion of daily exposures by the inhalation technical staff.

Whole body exposure chambers will be operated at an air flow to maintain approximately 15 air changes per hour. Air for the whole body exposure chambers will be drawn from humidified HEPA filtered room air except for group 4. Group 4 (low humidity group) will be supplied non-humidified HEPA filtered air from the Delmonox air purifier system. The temperature will be maintained at 20-24 degrees C and the humidity will be maintained at 40-60 %RH except for group 4 which will be maintained at 0-10 %RH.

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Feed and Water

Animals will have unrestricted access to certified feed (Purina Rodent Chow #5002, presented as pellets) and distilled water. No feed or water will be available to animals in groups 1,2, and 3 while they are held in nose only exposure tubes. Animals in groups 1,2, and 3 are expected to be held in nose only exposure tubes for 6.5 - 7 hours a day on each of the five exposure days. Feed will be withheld overnight prior to necropsy. Chemical analysis of feed, water, or bedding will not be performed because it is unlikely that contaminants would adversely affect the study.

F. In Life Data Collection from Experimental Animals:

1. Experimental Induction of Middle Ear Induction

Animals in groups 1,2, and 3 will have a serous middle ear effusion induced experimentally in the left ear according to the method of Goldie and Hellstrom, 1986). This treatment will occur once per day for the three days immediately preceeding start of exposures. The experimental procedure involves inducing anesthesia using Ketamine 50 mg/kg IM and Xylazine 10 mg/kg IM. The pinna of the ear is held with a small padded vascular clamp at an angle so that air at 12-14 degrees C at 2.5 liters/minute can then be directed into the ear canal for 20 minutes to expose the tympanic membrane to cold air. Little discomfort or pain is expected to occur as a result of this procedure. No analgesic agent will be administered because any pain or discomfort will have subsided before the rat regains consciousness. The rat will be observed after the procedure and returned to the cage after regaining sternal recumbency.

This procedure will be conducted by Hugh Lovejoy, M.D., of the Ear, Nose and Throat Department of the Bowman Gray/Baptist Hospital Medical Center. Dr. Lovejoy is a third year resident in the Ear, Nose, and Throat department and has been trained in this procedure by professional staff in his department.

2. Examination of the Ear

Prior to beginning the exposure phase of the study, each animal in all groups will be examined visually with an operating microscope for the presence or absence of middle ear effusion. Tympanometry will be performed with a model 83 American Electromedics Corp. Impedance Audiometer. These examinations will be done under Ketamine and Xylazine anesthesia with dosages of 50 mg/kg IM and 10 mg/kg IM, respectively. These examinations should take no more than 10 minutes per animal and should not cause significant discomfort. Visual ear examinations as well as tympanometry, will be performed daily on animals in groups 1,2, and 3 at the end of the daily exposure. The data regarding presence or absence of effusion and tympanogram type in left and right ears will be recorded on data flow sheets for each animal within the group. Animals in groups 4 and 5 will be examined at the start of the

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exposure phase of the study, after the third exposure day, and at the end of the fifth exposure day. Prior to necropsy, which will be conducted within 24 hours after the end of the last exposure, all of the animals in all groups will again be examined using the same method.

3. Viability Checks

Viability checks will be made twice daily on animals in room #47 after delivery and until the beginning of the exposure phase of the study. Rats that are allocated into experimental groups will be moved into whole body exposure chambers after release from quarantine. After beginning inhalation exposures, viability checks will be conducted once in the morning period during animal husbandry activities by the Animal Care staff. A viability check will be conducted daily in the afternoon after completion of exposures by the inhalation technical staff. A detailed clinical observation will be conducted by Animal Care staff after the fourth day of exposure within 1 hour after exposure. Moribund animals will be anesthetized with 70% CO_2 in air and exsanguinated via the vena cava. The carcass will be refrigerated at 3-5 degrees C until a necropsy is conducted by a veterinary pathologist or Principal Investigator. Any early deaths in the study will not be replaced by extra animals.

4. Dosimetry

Samples of blood will be collected from 5 rats in each of groups 1,2 and 3 after the fifth day of exposure within 10 minutes after termination of exposure. Rats sampled will be numbers 101-105,201-205, and 301-305. A sufficiently large volume will be collected for the analysis of both carboxyhemoglobin and plasma nicotine. Blood will be collected by personnel from the Animal Biology Division experienced in collecting blood from the retro-orbital sinus. Blood will be drawn from the retro-orbital sinus with heparinized micropipettes after the animal is anesthetized with 70 % CO_2 in air. Blood will be held on ice in plastic cuvettes containing disodium EDTA during the time between sampling and analysis. Blood carboxyhemoglobin concentrations will be determined on 0.5 ml of the total sample using a Model 482 CO-Oximeter(Instrumentation Laboratories, Hartford,CT.). Sub-samples of the blood will be prepared for the analysis of plasma nicotine and cotinine by the method of Davis (1986).

5. Collection of Body Weights

During the quarantine period, body weights will be determined upon delivery and every third day thereafter until the animals are allocated into experimental groups. Animals that are allocated into experimental groups will then be weighed 1 day prior to beginning the first inhalation exposure. Collection of body weights during the quarantine period and prior to the first exposure will be the responsibility of Animal Care staff.

During the exposure phase of the study, body weights will be collected daily in groups 1,2, and 3 after termination of exposure.

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In groups 4 and 5, body weights will be determined after the end of the third exposure and at the end of the fifth exposure. Body weight determination during the exposure phase of the study will be the responsibility of the principal investigator.

6. Survival Surgery
No survival surgery will be conducted in this study.

7. Experimentally induced pain or distress
Three procedures used in this experiment could produce minimal pain or distress in the experimental subjects. The first procedure is collection of blood from the retro-orbital sinus. This procedure is widely used in rodent studies and is the preferred method for collection of small volumes of blood. Minimal pain is involved in this procedure if conducted by qualified personnel. Only personnel trained in this procedure are allowed to take blood samples by this method. The rats are anesthetized with 70 % CO_2 for retro-orbital sinus bleeding to minimize any perception of pain or distress. No post-procedure pain is expected, therefore, no analgesic agent will be administered.

The second procedure which may involve minimal pain or distress is induction of middle ear effusion. The rats will be anesthetized with Ketamine 50 mg/kg IM and Xylazine 10 mg/kg IM during this procedure. The pinna of the ear is held with a padded vascular clamp and pulled to the side while cold air at 12-14 degrees C at 2.5 liters/minute is directed into the external auditory canal onto the tympanic membrane for 20 minutes. No post-procedure pain is expected so therefore, no analgesic agent will be administered.

The third procedure which may involve minimal distress is ear examination and tympanometry. Ear examination is conducted visually using a 2 mm speculum and a 40 X microscope. Tympanometry involves gently placing a padded transducer into the ear canal while transmitting a low frequency tone against the tympanic membrane. Another portion of the transducer senses the reflected sound waves and characteristic patterns are produced by the normal or abnormal ear. No pain is expected to be induced during this procedure and no analgesic agent will be administered. Anesthesia will be induced for this procedure using Ketamine and Xylazine as mentioned above to keep the animal immobilized during this observation.

8. Prolonged restraint
Rats in groups 1,2, and 3 will be restrained in nose only exposure tubes for 6.5 - 7 hours a day during the five exposure days. These nose only exposure tubes are specifically designed to minimize any distress to the animal. These tubes are designed so that the rat quickly adapts to the shape of the tube and becomes relaxed in the tube with no external evidence of distress. The animals do not vocalize during placement into the tubes or during the time they are restrained in the tubes. Previous studies have shown that rats

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restrained in these tubes for 6 - 7 hours a day do not lose body weight indicating that distress is not induced (Body weight curves from study TOX 34) by prolonged restraint.

VIII. EXPOSURE REGIMEN

The test material is sidestream smoke for 1R4F cigarettes (Tobacco and Health Research Institute, Lexington, KY.).

Aerosol Concentrations

The target WTPM concentrations are 0.1 mg/m^3 (group 2) and 1 mg/m^3 (group 3). WTPM concentration will be determined gravimetrically using 25 mm Teflon membrane filters.

Animal Exposures

Rats in all groups will be exposed for 6 hours/day for 5 consecutive days as indicated in the experimental design in Section VI.

Inhalation Exposure System

An AMESA smoke generator will be fitted with a stainless steel SSS collection cone. For groups 2 and 3, SSS will be drawn into a common plenum using 3" (76.2 mm) diameter PVC tubing. Humidified HEPA filtered dilution air mixed in an appropriate ratio with SSS from the plenum will be pulled through the whole body exposure chambers at a flow rate of approximately 15 CFM. In all exposure groups, temperature and humidity of the aerosol will be monitored with a condensation dew point hygrometer (Model 1100 DP, General Eastern Instrument Co., Watertown, MA.). A single smoke generator will provide test material for both groups 2 and 3.

The whole body chamber for group 4 (low humidity group) will be supplied with non-humidified HEPA filtered air from the Delmonox air purification system to provide approximately 15 CFM flow through the chamber. The whole body chamber for groups 1 and 5 will be supplied humidified HEPA filtered room air at a flow rate of approximately 15 CFM.

On exposure days, animals in groups 1,2, and 3 will be taken from their cage in the whole body chamber, placed in a nose only exposure tube, and replaced into the same cage position. The ventilation tubes on the tubes will be covered with duct tape. The position of the racks within the chambers will be changed daily to minimize any impact of rack position. Cages on the upper racks will be moved to the next lower rack on subsequent days of exposure and those on the bottom rack will be moved to the top position.

Pre-Exposure Characterization

Before the animal exposures begin, satisfactory achievement

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of uniformly distributed concentrations at or near the target concentrations will be confirmed.

Daily Characterization of Inhalation Exposures

Groups 1,2, and 3. During animal exposures probes inserted into the whole body exposure chambers will be used to monitor the aerosol presented. Gravimetric estimates of WTPM concentration will be determined with 25 mm Teflon membrane filters. On line monitoring of WTPM concentration will be accomplished with a RAM-1 aerosol monitor. Measurement of aerosol particle size in group 3 will be determined with a cascade impactor (In-Tox Products, Albuquerque, NM) twice during the study. Concentration of aerosol in group 2 is too low to permit particle size analysis by the cascade impactor.

Groups 1,2,3,4 and 5. Carbon monoxide and carbon dioxide concentrations will be analyzed with instruments (Horiba PIR-2000, Horiba Instruments, Irvine , CA.) calibrated daily with certified gases. Oxygen concentration will be determined with a Horiba PMA-200 instrument, also calibrated daily with a certified gas. Ammonia will be determined with a handheld monitor (GasTech, NH-275, Gas Tech, Newark, CA.) connected to a stainless steel probe inserted into the chamber. Very low concentrations of CO will be analyzed with daily calibrated Miran 80 gas analyzer (Foxboro Instrument, S. Norwalk, CT.). Data from on-line instruments will be logged manually every 60 minutes.

IX. NECROPSY AND HISTOPATHOLOGY

Animals from all groups will be killed within 24 hours after termination of the fifth day of exposure. At necropsy, animals will be weighed and then killed by first anesthetizing with 70 % CO_2 in air and then exsanguination via the vena cava.

Following euthanasia, tympanocentesis will be performed through the tympanic bulla to assess the viscosity of any middle ear effusion. The heads will be removed and the brain tissue removed as well. The temporal bones will then be harvested and fixed in 20 % formalin solution. The temporal bones will then be processed and cut in a manner to permit visualization of the mucosa. The tissues will be stained with hematoxylin and eosin and duplicate slides will be stained with periodic-acid-schiff (PAS) stain to facilitate evaluation of mucous secreting cells. Scanning electron microscopy will be conducted on the eustachian tube mucosa on three animal in groups 1,2, and 3 by RJR staff. In addition to collection of tissues from the middle ear, the larynx from each animal of each group will be taken for histopathological examination. The slides will be reviewed by Veritas Laboratories and the principal investigator in addition to a pathologist on staff at Wake Forest University Medical Center.

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X. STATISTICAL ANALYSIS

Appropriate statistical analyses on the induction of effusion, rate of clearance of effusion, qualitative effusion differences, as well as histopathological changes will be performed using appropriate statistical methods by Dr. Tim Morgan, a statistician at Wake Forest University Medical Center.

XI. QUALITY ASSURANCE

All data pertinent to this experiment will be recorded and kept for review so that the experiment could be reproduced.

XII. REPORTING

An interim report will be prepared following necropsy. This will include data on micro ear exams and tympanograms that were obtained during the study.

The final report will be prepared in draft form following completion of the review of histopathology. The final report will include:

- objectives and procedures as stated in the protocol
- description of the inhalation chambers and their operating conditions and performance
- tabulation of response data
- a separate pathology report, including tabulated gross and microscopic pathology
- statistical analyses and any conclusions developed from these data.

XIII. PROJECTED TIMING

Attempts will be made to achieve the following target dates (1991):

Delivery of animals: 17 January

Quarantine: 17 January - 31 January

Induction of middle ear effusions: 1 February - 5 February

Exposure starting dates: Group 1: 6 February
Group 2: 7 February
Group 3: 8 February
Group 4: 9 February
Group 5: 10 February

Necropsies: Group 1: 11 February
Group 2: 12 February
Group 3: 13 February
Group 4: 14 February
Group 5: 15 February

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