United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	**Cardiovascular and metabolic impairments from ozone exposure in rats: Impact of omega-3 and omega-9 fatty acid enriched diets.**
LAPR Number:	**17-12-002**
Principal Investigator	**Exemption 6**
Author of this Document:	**Exemption 6/RTP/USEPA/US**
Date Originated:	**11/19/2014**
LAPR Expiration Date:	**12/31/2017**
Agenda Date:	**01/14/2015**
Date Approved:	**02/03/2015**
Date Closed:	**03/21/2018**

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	02/03/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US by Exemption 6RTP/USEPA/US	02/03/2015	DMR approves	

Administrative Information

1. Project Title (no abbreviations, include species):
Cardiovascular and metabolic impairments from ozone exposure in rats: Impact of omega-3 and omega-9 fatty acid enriched diets.

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
Air, Climate Change and Energy program. NMP-4: Susceptible populations exposures to pollutant mixtures. Task ACE 120: Identify strategies to reduce the adverse health effects of air pollutants on susceptible individuals.
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/EPHD/CRB/[redacted]2014-001

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** [redacted]RTP/USEPA/US	EPHD ***Branch*** CRB	MD

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** [redacted]/RTP/USEPA/US	EPHD ***Branch*** CIB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in <u>non-technical language</u> such that it is understandable by non-scientific

persons. <u>Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research.</u> If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

The objective of this study is to determine whether the cardiovascular and metabolic effects caused by exposure to ozone can be blunted by supplementation with omega-3 fatty acids or olive oil. We will use healthy Wistar Kyoto (WKY) rats as an animal model.

Pulmonary health effects of ozone exposure has been well known but the cardiovascular and metabolic health effects of ozone are still under investigation. Although air pollution regulations have resulted in dramatic decreases in ozone levels over the past few decades, there are still tens of millions of people in the U.S. exposed to levels of ozone that are higher than the current Environmental Protection Agency standards. If dietary supplements or pharmacological agents can blunt the adverse health effects of ozone exposure, populations residing in highly polluted areas might be afforded some protection. Dietary supplements such as olive oil and fish oil have been shown to have anti-oxidant and anti-inflammatory effects that might offer protection against ozone exposure. A number of studies conducted over the last three decades have shown that the omega-3 polyunsaturated fatty acids (FA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can reduce the risk of cardiovascular events after myocardial infarction, as well as reduce atrial fibrillation recurrences in patients with persistent atrial fibrillation. Some components of olive oil, such as polyphenols and oleic acid (omega-9 FA) have been shown to have beneficial effects on endothelial function. In general, omega-3 FA and olive oil have several cardioprotective benefits, including suppression of arrhythmias and modulation of autonomic function, as well as anti-thrombotic, anti-inflammatory and vasodilatory effects. Moreover, ozone has been recently shown to induce metabolic impairments (glucose intolerance, insulin resistance, and insulin insensitivity) but it is not known if these effects of ozone can be blunted by omega-3 FA and olive oil containing diets.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of rat models is necessary in order to examine complex mechanisms associated with cardiopulmonary and metabolic interactions. In vitro culture studies will not allow one to examine the role or circulating biomarkers in inducing injury to extra pulmonary organs.

b. Justify the species requested:

National Institute of Health guidelines recommends the use of rats to study human cardiovascular and metabolic diseases. Rat has been also a preferred animal model for the study of cardiovascular injury from air pollution. Moreover metabolic disorders are better modeled in rats than in mice or in lower vertebrates. There are adequate genetic and protein data base for rats and reagents available to study cardiovascular effects. Toxicology data are available for rats to correlate findings of air pollution health effects. We have done a number of toxicological and metabolic studies using WKY rats. Since proposed studies involve examining cardiovascular and metabolic health effects, we propose using these WKY rats to understand how metabolic susceptibility relate to ozone exposure.

3. How was it determined that this study is not unnecessary duplication?

Literature searches in PubMed, and the peer reviewed toxicology and clinical literature were conducted on November 4, 2014 provide the evidence that proposed studies are not duplicative. Search terms include the following: omega-3 fatty acids, olive oil, cardiovascular and metabolic effects of ozone exposure, rats. No ozone metabolic studies have been done in Wistar rats based models to understand interaction of metabolism and ozone induced cardiovascular impairment. Also, no studies have examined the effects diets enriched with fish oil or olive oil on ozone induced cardiovascular and metabolic impairment and no animal studies have evaluated the dietary intervention strategies to mitigate the health effects of ozone exposure. We have the current knowledge, through direct interaction with scientists in the field and attending scientific meetings, and believe that our experiments are not duplicated anywhere else.

<u>SECTION B - In Vivo Procedures</u>

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

The following experiments are proposed for examining the ozone-induced cardiovascular and metabolic effects and evaluating the efficacy of fish oil or olive oil supplementation on mitigating these health effects using rat models.

Male WKY rats (21 day old) will be acclimatized for 1 week after their arrival, and then we will randomly assign into four groups (n=64 per group). Each group of rats will be fed with different diets (Purina Rat Chow 5001, TD.140728 6% coconut oil diet, TD.140727 6% olive oil diet, or TD. 140729 6% fish oil diet) for 8 weeks. The target animal weight will be around 350 gram at the end of dietary supplementation. At the end of the dietary supplementation, rats will be exposed to filtered air (n=32) or 0.8 ppm of ozone (n=32) for a period of 6 hours. Necropsies will be performed immediately after and 20 hours after exposure.

Hearts will be perfused to evaluate the cardiovascular function after ozone exposure and the mitigation efficacy of dietary supplementations. Immediately after and 20 hours after filtered air or ozone exposure rats (n=8 per group) will be i.p injected with pentobarbital and phenytoin mixture. Heparin (100 units) will be injected intravenously before removal of the heart. The hearts will be rapidly excised and the aortas will be cannulated. Hearts will be retrograde perfused. This procedure is categorized as Category D.

Metabolic effects of ozone exposure and the efficacy of dietary supplementation on mitigation of the metabolic effects will be examined by Glucose tolerance testing (GTT) and Insulin tolerance testing (ITT), and Insulin sensitivity evaluation tests. These tests in this study are categorized as Category C.

Glucose tolerance testing (GTT): Half of the rats assigned to metabolic assessment (18-hour time point; 64) will undergo GTT for a maximum of 3 times and ITT (see below) one time during the course of experiment (see attached ppt file for the protocol). Rats will be fasted for up to 8 hours but not water restricted. If GTT or ITT follows the exposure (during which food and water are restricted), rats will be allowed water during testing and food immediately following testing (in many cases fasting will occur during exposure periods prior to testing). We have done GTT in several of our previous studies occasions. The test works well and causes only momentary stress. In our experience performing GTT up to 5 times cause no peritoneal abnormalities as determined at the time of necropsy. In this testing once baseline glucose is measured, pharmaceutical grade glucose will then be injected intraperitoneally (maximum of 2 grams/kg body weight/10ml) and blood glucose will be measured at 30 min interval for four times in each rat. So each rat will have a total of 5 glucose measurements, each requiring 0.6 microliter blood for glucometer for testing (baseline plus four times following intraperitoneal injections of glucose) during each tolerance test. The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) form Sigma Aldrich. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection.

Insulin tolerance testing (ITT) in rats: Half of the rats assigned for metabolic assessment (0hr time point, 64 rats) will undergo ITT twice in addition to one ITT for 18hr time point animals (stated above). For ITT, instead of glucose, insulin is injected intraperitoneally and glucose measurements are done more frequently. The HumulinN which comes as solution of 100 Units/mL, will be diluted with injectable sterile saline to achieve 1U/mL dose for injection. This will be prepared fresh at each testing. Generally 0.75 to 1.5 U/kg insulin is injected during ITT in rats. We will use 1.0 U/kg insulin dose and only 6-7 hours fasting to avoid significant hypoglycemia during testing. Insulin injection is not likely to drop glucose level below 50 mg/dL based on the literature review, however, in case if blood glucose levels approach 40 mg/dL or below, a bolus intraperitoneal injection of pharmaceutical grade glucose solution (used in our GTT protocol) 2g/10mL/kg will be given to rats. The ITT rats will be a different subgroup than the GTT rats. No deleterious effects are expected during ITT.

Insulin sensitivity evaluation: Rats assigned for 18 hour time point (64 rats) will be intraperitoneally injected with 1.0 U/kg body weight dose of HumulinN, 10 minutes prior to necropsy. In order to determine insulin signaling changes, insulin injection in rats, right before their necropsy, is needed to allow for detection of changes in phosphorylation of proteins in liver and muscle, and if prior ozone exposure impacted this response. This will allow us to measure changes in insulin signaling in collected tissues after an ozone exposure. The preparation of diluted HumulinN and the intraperitoneal injection will be identical to the

protocol described in ITT testing. No follow-up glucose measurements will be done in these animals. This protocol is also used in many studies involving examination of insulin resistance and no deleterious effects are expected.

We like to emphasize that a highly conservative experimental design is adapted which will allow us to look at nearly 50 different biomarkers. We also will be sharing tissues with other investigators (brain and plasma) in order to reduce the use of animals. Another example of effective use of animals is that we collect blood for all clinical markers from same animal for which lung tissues are used for injury/inflammation assessment. Moreover, numerous molecular markers are examined in different tissues from same animal. We will also bank various tissue samples for potential future use.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

We propose to use 16 rats per diet group (8 for cardiovascular assessment and 8 for metabolic assessment) for all exposures that is minimally required for statistical evaluation. Since cardiovascular assessment will need to be done using ex vivo cardiac perfusion, the whole heart will need to be removed from rats immediately following heparin injection in vena cava. Thus, blood sample is not available to do metabolic assessment from same animals. Moreover, for metabolic assessment rats will need to be injected with insulin to determine insulin stimulated changes in the markers of glucose uptake in peripheral tissues. Thus, two groups of animals will be required for each exposure/diet condition. Eight rats per group are requested for adequate statistical analysis using standard analysis techniques. We have proposed to use minimum number of cage controls (8 per group; n=4 for cardiac assessment and n=4 for metabolic assessment) because exposing animals in wire mesh cages might cause some stress response. Determining this wire mesh cage related effect will be critical in the study because stress can induce cardiovascular and metabolic changes. So we need to determine if these exposure conditions contributed to observed metabolic changes from pollutant exposures not from stress.

This will require a total of 256 WKY rats to determine effects of dietary supplementation. We additionally request 8 rats for metabolic profile assessment prior to the start of dietary regimen. Since housing rats in the chamber during exposure might produce a stress response, we will have 8 additional rats on each diet maintained as a separate control group (cage controls) to compare to air control group (4 x 8 = 32 total cage control rats). Thus, a total of 296 rats will be used for this experiment (16 rats from each diet/exposure group; 8 for cardiovascular assessment and 8 for metabolic assessment (n=16/group) x 2 exposures x 2 time points x 4 diets). See attached Table 1 about the numbers of rats in each group.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	152	
D) Potential pain/distress relieved by appropriate measures:	144	
E) Unrelieved pain/distress:	0	

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery

☒ Food and/or water restriction (>6 Hours) ☒ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

Fasting>6 hours is required for the glucose tolerance testing and insulin tolerance testing. Animals will be monitored during fasting period and health status of animals will be tracked and records maintained on notebook. Non-survival surgery is required to examine the cardiovascular responses of ozone exposure ex vivo. Animals will be monitored during surgical procedure and the health status of animals will be tracked and record maintained.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
1). Dietary supplementation.
Four groups of rats (n=64 per group) will be fed with the following diets for 8 weeks: Purina Rat Chow 5001, TD.140728 6% coconut oil diet, TD.140727 6% olive oil diet, or TD. 140729 6% fish oil diet. The special custom made diets will be purchased from Harlan Laboratories.

At the end of supplementation, rats will be exposed once to either filtered air or 0.8 ppm ozone for a period of 6 hours and necropsy will be performed immediately after and 20 hours after filtered air or ozone exposure.

2). General whole body inhalation exposure conditions
During ozone inhalation exposures of maximum of 6 hours/day (whole-body), rats are placed in individual stainless steel wire mesh cages, and food and water are withheld while the rats are being exposed. The rats are weighed daily following exposure during exposure days and examined for any visible clinical signs of discomfort or poor health. The rats are also checked after each exposure when they are returned to home cages. All findings are recorded. Ozone exposures will be done using whole body exposure system.
b. Survival Blood Collections (method, volume, frequency):
Glucose tolerance testing (GTT) and insulin tolerance testing (ITT) will involve blood testing for glucose by tail prick as described below in Section B #5c. This requires <1 micro liter volume of blood at each time. This will occur for all animals assigned for GTT and ITT.
c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Table 1 presents the dietary supplementation regimens and the numbers of animals in each group. Eight rats in each group will be used for the metabolic study that include the following tests.

1). Respiratory monitoring using whole body Plethysmography - EMKA system: Selected group of rats from each experiment will be monitored for breathing parameters using Buxco system. Breathing parameters are monitored in freely moving rats. No restrain is used. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause before and after exposures. The rats are placed in a whole-body plethysmography for 5-10 min. We have routinely used this duration for acquisition of EMKA parameters which has been adequate for stabilization and recording.
No restraint or other stresses are involved in this process. This testing will be done prior to the first exposure, and after exposure at selected times. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

2) GTT protocol: For each test, the baseline blood glucose levels will be measured following 8 hours fasting. Since this food restriction technique is commonly used, we do not expect any deleterious effects of 8 hour food restriction (water will be provided), unless the animals encounter some unexpected health problem. We will take the usual steps of intervention by contacting the attending veterinarian and removing animals from the restricted treatment if needed. Blood will be collected by pricking the tip of the tail with a 25 gauge sterile needle following wiping with an alcohol swab and clean dry gauze. About 1 microliter blood droplet will be brought into contact with the glucometer strip (attached to Bayer Contour Glucometer) (0.6 micro liter of blood is aspirated in the strip). Glucose is measured within three seconds and recorded. After collection the prick area will be wiped with clean gauze. Once baseline glucose is measured, pharmaceutical grade glucose will then be injected intraperitoneally (maximum of 2 grams/kg body weight/10ml) and blood glucose will be measured at 30 min interval for four times in each rat. So each rat will have a total of 5 glucose measurements (baseline plus four times following intraperitoneal
injections of glucose) during each tolerance test. The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) form Sigma Aldrich. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection.

3) Insulin tolerance testing (ITT) in rats: Rats will be fasted for 6 hours and fasting glucose measurements will be taken. Then rats will be injected with 1.0 U/kg body weight HumulinN (injectable human insulin-recombinant, Veterinary grade) and blood glucose will be measured at 15, 30, 60, 90 and 120 minutes. This protocol has been extensively used for rats. GTT allows one to measure the capacity for peripheral glucose uptake by tissues while

ITT allows testing for hypothalamus pituitary adrenal axis performance. Generally both tests are performed in metabolic studies. Since we are investigating the role of hypothalamus pituitary adrenal axis in ozone-induced changes in insulin mediated peripheral tissue glucose uptake, we need to incorporate this test in our studies. The HumulinN which comes as solution of 100 Units/mL, will be diluted with injectable sterile saline to achieve 1U/mL dose for injection. This will be prepared fresh at each testing. Generally 0.75 to 1.5 U/kg insulin is injected during ITT in rats. We will use 1.0 U/kg insulin dose and only 6 hours fasting to avoid significant hypoglycemia during testing.

4) Insulin sensitivity evaluation: Rats assigned for ITT protocol (64 rats) will be intraperitoneally injected with 1.0 U/kg body weight dose of HumulinN, 10 minutes prior to necropsy. In order to determine insulin signaling changes, insulin injection in rats, right before their necropsy, is needed to allow for detection of changes in phosphorylation of proteins in liver and muscle, and if prior ozone exposure impacted this response. This will allow us to measure changes in insulin signaling in collected tissues after an ozone exposure. The preparation of diluted HumulinN and the intraperitoneal injection will be identical to the protocol described in ITT testing. No follow-up glucose measurements will be done in these animals. This protocol is also used in many studies involving examination of insulin resistance and no deleterious effects are expected.

In the above tests, each blood pricking will draw about 1 microliter blood. The total volume bled during one test will not exceed 20 microliters.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

n/a

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

n/a

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

The rat will be identified by numbering on tail. The animal will be weighed once per week. During exposure Exemption 6 Exemption 6 Exemption 6 **Exemption 6** will monitor animals, at least once per hour for entire exposure duration and the morning after exposure prior to necropsy. During non-exposure periods and the dietary regimen on each workday (include weekends and holidays), rats will be monitored once daily for visible signs of discomfort by Exemption 6 Exemption 6 Exemption 6 **Exemption 6Exemption 6** Ozone exposure protocol will occur only during working days. No weight loss or significant distress is expected in any of the experimental conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

n/a

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

n/a

c. Testing methods:

n/a

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

n/a

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

n/a

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

n/a.

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

n/a

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

Non-survival surgery to recover beating hearts for Langendorff procedure. Lab bench is wiped down with ethanol, covered with clean, new, absorbent pads. Instruments are clean. Area is clean and un-cluttered. Surgeon wears gloves. Animal is administered 150- 250 mg/kg pentobarbital solution IP, total volume to be delivered approximately 1 ml. (Luer lock syringe used). This is delivered intraperitoneally after dilution with saline by half. New 25 ga (or larger gauge) needle (new per each rat) is used. Each rat is monitored for loss of righting response. When this occurs, they are tested for a surgical plane of anesthesia by multiple firm toe pinches to hind feet toes. If no response is seen, after proper plane of anesthesia is reached, the animal's fur is clipped and surgical area wiped clean of fur with alcohol. If rat begins to respond to surgical stimuli, procedure is halted, further doses of pentobarbital (0.5 mL) are delivered IP, and animal is monitored for lack of response. Monitoring for surgical plane of anesthesia before opening the thorax includes monitoring closely for lack of response to multiple firm toe pinches, lack of response to tail pinch, and lack of response to mid-chest pinch. If no response, then proceed with incision to open thorax. The inferior vena cava is injected with 0.2 mL diluted heparin (100IU diluted with saline 1:1), to prevent clotting to prepare for isolated heart reperfusion technique. Recheck for anesthetic plane. Heart removed, suspended in buffer.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

n/a

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

n/a

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

n/a

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

n/a

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

No deleterious effects are expected from dietary supplementation, ozone exposure, and metabolic testing (GTT, ITT) in rats. Transitory weight loss or dehydration may occur after ozone exposure. Rats will be weighed once per week during the dietary supplementation period and before and after ozone or filtered air exposure. Attending Veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 20% or greater from dietary supplementations or ozone exposure.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

No deleterious effects are expected from dietary supplementation, ozone exposure, and metabolic testing (GTT, ITT) in rats. Transitory weight loss or dehydration may occur after ozone exposure. Rats will be weighed once per week during the dietary supplementation period and before and after ozone or filtered air exposure. Attending Veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 20% or greater from dietary supplementations or ozone exposure.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful

procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

This study will evaluate the cardiac functional changes (heart rate, developed pressure, and cardiac contractility) after ozone exposure and these measurements require beating hearts. In order to keep the hearts beating it requires that the Langendorff isolation procedure which takes less than one minute from opening the chest to mounting the hearts to the perfusion setup. The procedure requires the heart to be isolated quickly when the rat is in deep anesthesia without feeling any pain. There is no alternatives for the Langendorff procedure that maintains hearts beating. PubMed has been used to search for the alternative in the Langendorff cardiac isolation procedure in November 2014, but alternatives were not found. The search covered years from 2014 backward and key words used were cardiac function, heart rate, cardiac contractility, left ventricular developed pressure Langendorff, cardiomyocytes, cardiac slices, beating hearts, rat.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:	296
b. Animals to be transferred from another LAPR:	0
LAPR Number that is the source of this transfer:	
c. Animals to be transferred from another source:	0
d. Offspring produced onsite (used for data collection and/or weaned):	0
e. TOTAL NUMBER of animals for duration of the LAPR	296

2. Species (limited to one per LAPR): Rat(s)

3. Strain: WKY rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

4. Sources of animals:

Charles River Laboratories Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Rats will be housed in one of the animal housing room upon arrival Exemption 6 Exemption 6 Exemption 6 - or other available room) and during non-exposure periods.
2. During exposure rats will be transferred in an original rack with rats housed in home cages to green floor inhalation exposure rooms (EMKA - whole body plethysmography; whole body exposures - Exemption 6 Exemption 6 Exemption 6). Once the exposure and measurements are complete rats will be transferred to their home cages in the same rack and moved back to animal holding room.
3. The day of necropsy animals will be transferred to Exemption 6 Exemption 6 for necropsy using transfer cages with beta chips bedding and filtered cage tops. The rats for the cardiac perfusion experiment will be transferred to Exemption 6 Exemption 6 using transfer cages with beta chips bedding and filtered cage tops.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

no ***Room Numbers:***

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)
none

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.
We request to use beta chips bedding with two times per week cage change schedule. This requirement of bedding will be specified with each animal order.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)
One week after rats arrive, we will request animal care staff to provide special diets with different color to be fed to animals and the color coded tags will be used on the cages to indicate diet. We will also have color coded labels placed on the cage card to match the food color as suggested. The animals will be weighed once per week by animal care staff and the request will be submitted for this assistance. The animal care staff will also be requested to monitor the daily food intake by weighing food daily for a period of two to three weeks after the dietary regimen to determine the caloric intake of each diet. We will submit a request for this assistance.

10. Housing and Enrichment.
The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.
All animals will be housed 2/cage with beta chips bedding on solid bottom cage in building A during non-exposure periods.
Enviro-dri® nesting material will be used in cages for enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1) Medical grade Heparin solution (100 units) will be injected into vena cava right before the hearts are isolated.

2) Medical grade pentobarbital dilute (150 mg/kg) will be injected i.p. for euthanasia.

3). Pharmaceutical grade D-glucose solution for GTT: no data are available on LD50 value for glucose in
MSDS obtained from Sigma Aldrich company. The osmolality of 20% glucose solution is 1110 mos/L. The intraperitoneal injection: the dose of glucose: 2g/kg/10ml.

4). Veterinary grade insulin solution will be injected i.p. with 1.0 U/kg body weight. Intravenous rat LD50 is 145 mg/kg.

5) Custom-made diet will be fed to rats for 8 weeks. Three diets will be purchased from Harlan Laboratories, 6% fish oil diet, 6% olive oil diet, and 6% coconut oil diet. Rat chow will be used as control

diet.
The quality of animal food will be monitored by following the use-by date, ensuring proper storage not go rancid. No overt effects of these diets are expected. All animals will be monitored during the dietary supplementation period.

6) Ozone exposure: Ozone exposure will occur in whole body exposure chamber to a maximum of 0.8 ppm concentration. The LC50 for ozone is 4.8 ppm in rats. Health and safety protocol for ozone is available and will be followed during exposure (HSRP #778; Title: Small Animal Inhalation Exposures to Nitrogen Dioxide; includes ozone protocol).
No overt toxicity is expected. All animals will be monitored continuously during exposure.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

yes

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

none

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

none

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols and oversee the experiment.	Exemption 6 has 20 yeas of experience working with rats at EPA and other institutions, all required training completed.
Exemption 6	Principal Investigator	Plan study, prepare protocols and oversee the experiment.	Exemption 6 has Twenty years of experience working with rats at EPA and other institutions, all required training completed.
Exemption 6	Post-Doc	Plan study and oversee the experiment. Assist in study planning,	Exemption Exemption Exemption has taken required animal training courses at EPA and has experience working with rats at EPA

		animal handling, testing and necropsy	
Exemption 6	Technical Staff	Oversee the experiment, perform plethysmography measurements. Assist in study planning, animal handling, testing and necropsy	Twenty years of experience working with rats at EPA and other institutions, all required training completed.
Exemption 6	Student	Assist in study planning, animal handling, testing and necropsy	Exemption Exemption Exemption Exemption has taken required animal training courses at EPA and has experience working with rats at EPA. Exemption 6 will continue training and monitoring Exemption 6 Exemption 6 Exemption 6 Exemption 6 animal handling work
Exemption 6 Exemption 6 Exemption 6 Exemption 6	Technical Staff	Oversee the experiment. Assist in study planning, animal handling, testing and necropsy	Twenty years of experience working with rats at EPA and other institutions, all required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals will be euthanized either immediately following last exposure or 24 hrs following last exposure

2. Describe the euthanasia techniques:

Method(s): Anesthesia plus exsanguination

Agent(s): Pentobarbital injectable preparations, diluted with sterile saline to achieve 200 mg/ml concentration

Dose (mg/kg): 150-250 mg/kg pentobarbital

Volume: maximum 3 ml

Route: intraperitoneal

Source(s) of information used to select the above agents/methods:

- Veterinary Staff
- IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

n/a

4. Describe how death is to be confirmed.

Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Euthanized by Animal Care Contractor

Transferred to another study

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	11/19/2014

Submitted: 12/01/2014

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*

Director Exemption 6	12/01/2014 by Exemption 6 Exemption 6/RTP/USEPA/US	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6/RTP/USEPA/US	EPHD ***Branch*** CIB	MD ***Submitted to Branch Chief for Approval*** 12/01/2014 11:46 AM

ATTACHMENTS

17-12-002 PI resp 1-29-15.pdf Diet study GTT and ITT protocolsR1.pptx Table 1-numbers of rats.docx

17-12-002 PI resp 1-7-15 details.pdf 17-12-002 PI resp 1-7-15.pdf

Actions

First Update notification sent: 11/04/2015
Second Update notification sent: 12/01/2015
First 2nd Annual notification sent: 11/02/2016
Second 2nd Annual notification sent: 12/09/2016
1st Expiration notification sent: 10/30/2017
2nd Expiration notification sent: 12/04/2017

History Log: