Study Title

Effect of Gamma Tocopherol Enriched Supplementation on Response to Inhaled O3 Exposure

NCT number	NCT02911688
Document Date	July 18, 2017

Protocol Version Date: July 18, 2017

Summary of Changes

1) 1) In Section A.3.1 under Inclusion Criteria, the definition of of a positive methacholone test (item d.) has been corrected to read "...a provocative concentration of methacholine of 10mg/ml or less producing a ***20%*** fall in FEV1...." (This was also corrected in the protocol itself.)

2) 2) The collection of samples of nasal epithelial cells (NEC) and epithelial lining fluid (ELF) is not done at the Screening Visit (V-0). Those procedures have been removed from the description of that visit in Section A.4.2., and now the Application matches the protocol.

Protocol Version Date: February 17th, 2017

Summary of Changes

1) Based on published data[1], the pulmonary function criteria have been relaxed, including:

   a. the minimum allowable volume in liters of forced expiratory volume in the first second (FEV1) has been decreased from 80% to 75% of predicted; and

   b. the FEV1/FVC (forced vital capacity) ratio will no longer be used for determination of eligibility because the value can be artificially low when a person has an exceptionally large FVC.

Our original restrictions on spirometry measures for study inclusion were very conservative. Since the approval of the original ozone IND, we have found that the mean drop (from that day's baseline values) in FEV1 is 13.6%, and mean drop in FVC is 11%. These changes are transient, evident immediately after subjects exit the chamber, and resolve to within 90% of that day's baseline by 4 hours after the end of the ozone exposure. For a subject to be released from the CEMALB after ozone exposure, their spirometry must show these measures to be within 90% of that day's baseline.

Inhaled allergen challenge requires a drop in FEV1 of 20% to be achieved, and presents a much longer period of bronchoconstriction, particularly in those with a late phase response (onset evident 3-7 hours after the initial drop in FEV1). Inclusion criteria for inhaled allergen challenge requires an FEV1 of at least 70% of predicted with no restrictions on FEV1/FVC ratios. Given that the ozone effects on spirometry are milder than those seen with inhaled allergen challenge, we feel that it is safe to slightly relax our spirometry criteria. However, our entrance criteria for FEV1 are still more conservative than those used by others with inhaled allergen challenge.

[1] Diamant, et al. Inhaled allergen bronchoprovocation tests. J Allergy Clin Immunol 2013; 132:1045-55

2) The Ozone Symptom Questionnaire score is removed from the list of inclusion criteria.

3) The consent form and the application have been revised to indicate the following changes:

   a. Now that several subjects have gone through the various visits, we are revising the estimated duration of some of the visits – some longer and some shorter. The estimated total duration has not changed

   b. The description of Study Visit 7 (Final Study Visit) now includes, "This visit will occur 5 to 10 days after your final ozone exposure, and ...."

   c. Section A.6.10 of the application has been revised to include new information, and in the consent form, *Risks associated with Vitamin E administration* now reads: "In a previous study, nausea, vomiting, bloating and/or diarrhea were

commonly reported. These symptoms generally occurred in the first day or two of dosing. You are encouraged to take the study treatment with food containing fat, such as cheese or peanut butter, to minimize these effects."

Protocol Version Date: January 13, 2017

Description of Change

Assessment of the impact of gamma tocopherol on inflammatory gene expression in peripheral blood mononuclear cells (PBMCs) is added to the protocol.

Protocol Version Date: October 28, 2016

Summary of Protocol Changes

1. Spirometry prior to exercise, vital sign measurement after exercise, and a pregnancy test (as applicable) have been added to the Training/ Baseline Visit (Visit 0a) to optimize subject safety.
2. "Use of investigational drugs within 6 weeks of screening" has been added to the list of exclusion criteria.
3. The Asthma Symptom Score has been removed from the list of inclusion criteria and from the list of assessments performed at Visits 0, 1 and 4; it has been renamed Ozone Symptom questionnaire at Visits 2, 3, 5, 6 and 7. (The questionnaire to be used has not changed, and is attached to the application.)
4. The target minute ventilation has been decreased from 25L/ min/ BSA(m^2) to 20L/min/BSA. It was decided that the lower rate is sufficient to assess the effect of ozone on the airway.
5. The duration of participation has been changed to "6 weeks to 5 months;" practically speaking, 6 weeks is about as little time as it would take for the participant to complete all visits.
6. A blood draw for clinical lab tests (CBC, PT/INR, aPTT and C-reactive protein [CRP]) and biomarkers has been added to the 24 Hr Post-Exposure Visits (Visits 3 and 6), to optimize subject safety and to add an extra timepoint for evaluation of the effects of γ-tocopherol on inflammation. (A sample for CRP has also been added to the Screening and pre-exposure lab assessments on Exposure Visits 2 and 5 to establish baseline values.)
7. The final follow-up/End of Study Visit (Visit 7) will include a blood draw for CBC, PT, and/or aPTT, "if [there were] abnormal values at Visit 6."

Protocol Version Date August 17, 2016

Summary of protocol changes:

1. Sputum induction - this procedure has been added to the treatment initiation visits of both treatment periods (initial and cross-over, Visits 1 and 4). The rationale for this addition is two-fold. the time between sputum inductions and the mucocilliary (MCC) scans will be approximately the same (48 hours) for each subject, and the extra specimens will provide more baseline data on the primary endpoint.

2. Blood pressure will be monitored during the 3-hour O_3 exposure, with measurements taken at least once every hour.

Protocol 4: Effect of gamma tocopherol enriched supplementation on response to inhaled O3 exposure

a. Objectives and Purpose.
To test the hypothesis that gamma tocopherol supplementation inhibits ozone induced airways inflammation in allergic asthmatics

b. Investigators, Facilities, and Institutional Review Board

Role in project	*Name and Address*	*Title*
Principal Investigator	David B. Peden, MD, MS CEMALB, 104 Mason Farm Road The University of North Carolina CB#7310 Chapel Hill, NC 27599-7310	Professor of Pediatrics & Director, Center for Environmental Medicine, Asthma and Lung Biology
Co-Investigator	Neil Alexis, Ph.D. CEMLB, 104 Mason Farm Road The University of North Carolina CB#7310 Chapel Hill, NC 27599-7310	Professor of Pediatrics University of North Carolina
Co-Investigator	Michelle Hernandez, MD CEMALB, 104 Mason Farm Road The University of North Carolina CB#7310 Chapel Hill, NC 27599-7310	Associate Professor of Pediatrics University of North Carolina
Co-Investigator	William Bennett, Ph.D CEMALB, 104 Mason Farm Road The University of North Carolina CB#7310 Chapel Hill, NC 27599-7310	Professor of Medicine & University of North Carolina

Curriculum Vitae for Drs., Peden, Alexis, Hernandez and Bennett are appended.

Facilities. Volunteers for these studies will be recruited, screened and undergo challenge procedures at the Center for Environmental Medicine, Asthma and Lung Biology, CB#7310, 104 Mason Farm Road, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7310. All necessary clinical research equipment, medical equipment, and laboratory equipment is located within the Center.

Institutional Review Board. This study will be reviewed and approved by the Biomedical IRB for the UNC School of Medicine, The University of North Carolina at Chapel Hill CB# 7097, 720 Martin Luther King, Jr. Boulevard, Chapel Hill, NC 27599-7097 prior to enrolling the first subject.

c. Patient selection criteria, exclusion criteria and numbers.

Up to 32 mild allergic asthmatic volunteers ages 18-45 will be recruited, to determine the comparative effect of 0.25 parts per million ozone (O3) in persons receiving 2 capsules of 700mg each gamma tocohpherol (yT) supplement for 48 hours. Subjects will undergo baseline measurements including venipuncture, induced sputum and mucociliary clearance. After 48 hours of treatment subjects will undergo O3 exposure, followed a few hours later by repeating the venipuncture, induced sputum and mucociliary clearance scan. Subjects will have a minimum 3 week washout and repeat the procedures, in a double blind, crossover fashion, with safflower oil as the placebo control.

There will no gender or ethnic restrictions. Spirometry will be performed to determine the current level of lung function. Women of childbearing potential will not undergo the methacholine challenge or mucocilliary clearance scans unless a urine pregnancy test confirms the absence of pregnancy.

Subjects for this study will be otherwise healthy, allergic asthmatic 18-45 year old males and females. Our recruitment will include up to 32 volunteers, and at least 50% female subjects with at least a 30% minority representation. It is anticipated that the demographics of our subject population will reflect those of the local university community. Subjects are recruited through a separate screening protocol, IRB # 98-0799, which is performed to identify subjects for potential participation in experimental challenge protocols such as this one.

Specific Inclusion Criteria:
a) Age 18-45 of both genders
b) Negative pregnancy test for females who are not s/p hysterectomy with oopherectomy
c) History of episodic wheezing, chest tightness, or shortness of breath consistent with asthma, or physician diagnosed asthma.
d) Positive methacholine test. A positive test is defined as a provocative concentration of methacholine of 10 mg/ml or less producing a **20**% fall in FEV1 (PC20 methacholine) by the method used in a separate screening protocol; OR physician diagnosed asthma with a history consistent with mild, intermittent asthma after age 6; OR a positive bronchodilator challenge. Subjects with regular use of inhaled corticosteroids may be included, these volunteers must be able to come off of the ICS for 2 weeks without increased symptoms or increased need for beta agonist rescue medication.
e) FEV1 of at least **75**% of predicted without use of bronchodilating medications for 12 hours or long acting beta agonists for 24 hours
f) Allergic sensitization to at least one of the following allergen preparations: (House Dust Mite f, House dust mite p, Cockroach, Tree mix, Grass Mix, Weed Mix, Mold Mix 1, Mold Mix 2, Rat, Mouse, Guinea Pig, Rabbit, Cat or Dog) confirmed by positive immediate skin test response; OR a history of seasonal or perennial allergy symptoms such as sneezing, or itchy and watery eyes.
h) subjects must be willing to avoid caffeine for 12 hours prior to all visits.
i) subjects must be willing to avoid non-steroidal anti-inflammatory medications for 4 days prior to all visits

Specific Exclusion Criteria:
1. Clinical contraindications:

a) Any chronic medical condition considered by the PI as a contraindication to the exposure study including significant cardiovascular disease, diabetes, chronic renal disease, chronic thyroid disease, history of chronic infections/immunodeficiency, history of tuberculosis
b) Physician directed emergency treatment for an asthma exacerbation within the preceding 3 months
c) Moderate or Severe asthma
d) Exacerbation of asthma more than 2x/week which would be characteristic of a person of moderate or severe persistent asthma as outlined in the current NHLBI guidelines for diagnosis and management of asthma.
e) Daily requirement for albuterol due to asthma symptoms (cough, wheeze, chest tightness) which would be characteristic of a person of moderate or severe persistent asthma as outlined in the current NHLBI guidelines for diagnosis and management of asthma. (Not to include prophylactic use of albuterol prior to exercise).
f) Viral upper respiratory tract infection within 2 weeks of challenge.
g) Any acute infection requiring antibiotics within 4 weeks of exposure or fever of unknown origin within 4 weeks of challenge.
i) Mental illness or history of drug or alcohol abuse that, in the opinion of the investigator, would interfere with the participant’s ability to comply with study requirements.
j) Medications which may impact the results of the O3 exposure, interfere with any other medications potentially used in the study (to include systemic steroids, beta antagonists, non-steroidal anti-inflammatory agents)
k) Any history of smoking in the year prior to study enrollment; lifetime smoking history > 10 pack years
l) Nighttime symptoms of cough or wheeze greater than 1x/week at baseline (not during a clearly recognized viral induced asthma exacerbation) which would be characteristic of a person of moderate or severe persistent asthma as outlined in the current NHLBI guidelines for diagnosis and management of asthma
m) Allergy/sensitivity to study drugs, or their formulations.
n) Known hypersensitivity to methacholine or to other parasympathomimetic agents
o) History of intubation for asthma
p) Unwillingness to use reliable contraception if sexually active (IUD, birth control pills/patch, condoms).
q) Abnormal PT or aPTT values at screening or during the treatment period. Normal values will be those published by the clinical lab (Labcorp, INC).
r) Any bleeding disorder
s) Orthopedic conditions which would prevent the volunteer from performing moderate exercise on a treadmill.
t) Radiation exposure history will be collected. Subjects whose exposure history within the past twelve months would cause them to exceed their annual limits will be excluded.
u) Recent nasal surgery

2. Pregnant women: Pregnant women will be excluded due to the radiation exposure associated with the mucociliary clearance scans. Pregnant women and children (<18 years as this is the age of majority in NC) will also be excluded since the risks associated with O3 exposure to the fetus or child, respectively, are unknown and cannot be justified for this non-

therapeutic protocol. Individuals over 45 years of age will not be included due to the increased possibility of co-morbidities and need for prohibited medications.

3. Use of the following medications:
a) Use of systemic steroid therapy within the preceding 3 months for an asthma exacerbation. All use of systemic steroids in the last year will be reviewed by a study physician.
b) Use of inhaled steroids, cromolyn or leukotriene inhibitors (Montelukast or Zafirkulast) except for use of cromolyn exclusively prior to exercise. Subjects who are prescribed ICS must demonstrate the ability to withhold these medications for 2 weeks prior to any visit without increased symptoms or increased requirement for beta agonist rescue.
c) Use of daily theophylline within the past month
d) Daily requirement for albuterol due to asthma symptoms (cough, wheeze, chest tightness) which would be characteristic of a person of moderate or severe persistent asthma as outlined in the current NHLBI guidelines for diagnosis and management of asthma. (Not to include prophylactic use of albuterol prior to exercise).
e) Use of any immunomodulatory therapy within the preceding 12 months
f) Use of beta blocking medications
g) Non-steroidal anti-inflammatory drugs in the 4 days prior to the baseline visit as well as 4 days prior to the pre-exposure days. Subjects will not be allowed to use NSAIDs during the treatment periods
h) use of any investigational agent within 6 weeks of Screening.

4. Allergy/sensitivity to study drugs or their formulations: Known IgE mediated hypersensitivity to tocopherols, albuterol, diphenhydramine or corticosteroids.

5. Physical/laboratory indications (may be temporary exclusions):
a) Abnormalities on lung auscultation
b) Temperature > 37.8
c) Systolic BP >150 mm hg or < 90 mm Hg or diastolic BP>90 mm Hg or < 50
d) Oxygen saturation of < 94%
e) Abnormal PT or PTT values at screening or during the treatment period. Normal values will be those published by the clinical lab (Labcorp, INC).

6. Inability or unwillingness of a participant to give written informed consent.

d. Design of Study.
This will be an intention-to-treat study. The effect of gamma tocopherol (yT) on O3 response of continuous variables (e.g., PMNs in sputum, cytokines, mucins and MCC) will be assessed using the difference (delta) from baseline (i.e. post –O3 minus baseline) values, comparing yT versus placebo treatment periods,

Potential subjects will be seen for an initial visit at which time informed consent will be obtained. Once the subject is deemed eligible by clinical lab and sputum values, they will return to the research lab for treadmill training and baseline muco-ciliary clearance (MCC) lung scan including a lung transmission scan. Treadmill training is required to determine the treadmill settings for the exposure day, and will be done prior to MCC to control for exercise

effects on the baseline MCC measurements. Baseline nasal endpoints will also be collected to determine pre and post O3 nasal epithelial cell gene expression levels and cytokine production. On the following day, subjects will return to the imaging lab for a 30-minute follow-up MCC scan.

Within 30 days of the screening visit, subjects will come in for randomization to placebo or gamma tochopherol (yT) supplement. At this visit, vital signs will be measured and spirometry will be performed, then subjects will undergo sputum induction. They will be instructed to begin the study drug the evening of the visit, then take it every 12 hours for a total of four doses (the last dose being taken the morning of O3 exposure). They will arrive on the exposure day and undergo baseline measurements including venipuncture, spirometry, and NO. Subjects will then undergo O3 exposure, followed by mucociliary clearance, venipuncture, and induced sputum. Subjects will return to the research lab the next morning for a 24 hour follow up. Subjects will have a 3 week-3 month washout and repeat the procedures during the second treatment period, with placebo or yT, depending on randomization.

Subjects will be queried at all visits for any unexpected or adverse events, general health status, medication and supplement use, and for any recent injections.

After completion of 48 hours of treatment, subjects will undergo baseline (pre O3) measures, including vital signs with symptom scoring, urine pregnancy test for women of childbearing potential, spirometry, venipuncture for CBC, PT, aPTT, and C-reactive protein (CRP), as well as study endpoints, up to about 50cc of blood. Subjects will then undergo exposure to 0.25ppm of O3 in a chamber, with intermittent exercise for three hours. Spirometry and symptom scoring will be performed immediately after exposure, and 30 to 45 minutes after exposure. Subjects will undergo (MCC) lung scan 1 hour after completion of exposure, followed by venipuncture for approximately 50cc blood, and by sputum induction (with albuterol pretreatment) and nasal samples at the completion of MCC.

The following day the subject will return for vital signs; symptom scoring; spirometry; venipuncture for CBC, PT, aPTT, (CRP), and study endpoints; and a 30 minute follow-up MCC scan.

Subjects will be sent home with a diary to complete each day for 4 days. A follow up phone call will be made to the subject within 7 days of completing the first O3 exposure to review the diaries, following a phone script (attached). If there are any health concerns from either the subject or the study team member, subjects will be asked to come to the research lab for evaluation.

Subjects will be given a minimum of 3 week, maximum of 3 month washout and then repeat the series with crossover study medication. Samples for analyses will be collected and the MCC repeated at the same time points as those used in the initial sequence

Subjects will return the next week after completing the second session for a check out visit which will include vital signs, symptom scoring, spirometry and if any values are out of the normal range, or if the subject has health symptoms or concerns, a physical examination and evaluation by a study physician.

Randomization will be determined by Dr. Haibo Zhou, and the randomization will be given to IDS. Randomization blocks will be n=4.

***Efficacy Measures**:*
We expect that treatment with yT will increase airway sputum PMN and eosinophil response to inhaled O3. We also expect similar effects of yT on all exploratory measures, especially mucin secretion and MCC consistent with our preclinical data. We will use regression modeling to assess the impact of the GSTM1 null genotype on response to O3 and efficacy of yT supplementation. Our published data on the effect of this GSTM1 null genotype on response to LPS indicates that n=32 volunteers will yield 15 GSTM1 null participants. We expect enhanced response to O3 in this subgroup and selective efficacy of antioxidant interventions in this group.

***Pharmacology measures**:*
We will also examine baseline and post treatment levels of alpha and gamma tocopherol and alpha and gamma CEHC (an active metabolite of tocopherol)

***Sample Size Estimates**:*
Sample size for induced sputum granulocyte response: Our primary endpoint will focus on the effect of γT on reducing ozone-induced neutrophilic inflammation, defined by neutrophils/mg of sputum (PMNs/mg). We are comparing the post-O3/pre-O3 ratio of log PMNS/mg as the data are not normally distributed. Using data from our study of the effect of 2 mg of Fluticasone Propionate (FP) on O3 response, we observed in the placebo group that the Post/Pre ratio in log PMNs/mg has a mean 1.205, and a standard deviation 0.225. In the treatment group, the post/pre ratio has a mean 1.005, and a standard deviation 0.206 If γT-mT is 50% as effective as 2mg FP, the correlation between active treatment and placebo responses is 0.5, and ß= 0.80 and α=0.05, then the estimated sample size is n=29. We have inflated the sample size to 32 for potential drop-outs seen with crossover studies.

Sample size for MCC: In a study of healthy subjects (n=16) we found a standard deviation of the delta between 2 repeat MCC measures of 8.7 % (SD) in average clearance through 90 minutes (Ave90Clr) (i.e. the average of %clearance ((1-retention) X100) over all 10 min data points from 10-90 min). In a study of 18 healthy subjects challenged with LPS in a repeated, crossover design, we observed a decrease from 13% to 8% Ave90Clr compared to no LPS. If yT treatment completely blocks O3 induced decrease in MCC, using an estimated SD of 8.7 % for repeat measures, a decrement of 5% in Ave90Clr (or a 40% decrement in MCC) should be seen with n=24 volunteers (ß= 0.80 and a=0.05). The overall sample size of 32 for the primary endpoint should provide sufficient power to detect changes in this secondary endpoint.

***Safety measures**:*
Criteria for safety within the entire protocol (failure of which would result in suspension of further study) will include the following:

1. No more than 20 % (n=6) of patients will fail the individual safety criteria outlined below. Safety data endpoints will be assessed after every 10 subjects, and all adverse events will be reported to CBER, the UNC IRB and NIEHS.
2. No occurrence of any Serious Adverse Event

Criteria for safety of a given individual following a specific challenge will include (failure of which would result in discontinuation of that subject from further study):

1. No greater than 40% decrease in FEV1 or FVC from pre- O_3 challenge values, which do not improve within the first 2 hours of challenge.

2. FEV1 must recover without treatment to within 90% of baseline within 3 hours of challenge without treatment. (Ibuprofen 800mg will be employed as rescue medication if the FEV1 does not return to 90% of baseline, or if symptoms persist.)
3. Specifically no symptom score greater than "moderate" (3 on a scale of 0-4) for shortness of breath or cough throughout the protocol and no assessment of severe (4 on a scale of 0-4) for any other individual item in the symptom score criteria.
4. Oxygen saturation of >93% throughout the exposure period and must be at least 2% of baseline measure.
5. If albuterol therapy is needed more than once during an observation period.
6. Any increase in total symptom score over baseline within 96 hours after challenge which are greater than 6 out of a possible 24.
7. Any significant abnormalities in PT and PTT during the treatment periods will be assessed and reported to CBER, IRB and NIEHS.

Protocol Outline:

I. SCREENING (Visit 0):

1. Vitals signs will be collected temperature, pulse, systolic and diastolic BP, respiratory rate, and SpO2 (oxygen saturation),
2. A brief physical exam by a study physician.
3. Urine pregnancy tests, using a commercially available testing device, will be performed on all women of childbearing potential. Pregnancy tests are considered valid for 7 days.
4. FeNO will be collected.
5. Spirometry will be performed following ATS/ERS standard procedures to obtain FVC and FEV1.
6. A standard 12 lead ECG will be obtained.
7. Venipuncture will be performed. CBC, PT/INR and aPTT, and CRP will be collected and sent to Labcorp, Inc. Additional blood will be collected for study endpoints, a total of about 50cc will be collected.
8. Subjects will undergo sputum induction. Subjects will be pretreated with 2-4 puffs of albuterol prior to sputum induction, and post albuterol spirometry will be collected. Subjects will inhale 3% saline for 7 minutes from an ultrasonic nebulizer. After the inhalation is complete, subjects will be asked to "cleanse" the upper airway by rinsing the mouth with water, gargling, clearing the throat, then blowing the nose. This is followed by a voluntary cough. Sputum will be placed into a sample cup. Spirometry will again be performed to ensure that the FEV1 does not fall more than 20% from the post albuterol value. The subject will then repeat the procedure with 4% and then with 5% saline. FEV1 must be within 5 % of baseline before a subject will be discharged from the research lab. If the sputum sample is adequate for required analysis, the subject is potentially eligible for continued study participation.

II. TRAINING (V0a/b):

All subjects who successfully complete the screening procedures will return to the research lab for a training visit, prior to V1. The purpose of this visit is to establish the treadmill settings required for the volunteer to breathe at 20 L/min/BSA in m^2 (DuBois). After baseline

vital signs the subject will be shown the treadmill and instructed in proper and safe use. The settings will be increased until the desired minute ventilation is reached. A urine pregnancy test will be repeated on those for whom it is applicable. Vitals signs, including temperature HR, RR, BP, and SpO2, will be collected before and after treadmill exercise. Spirometry will be performed before exercise begins. Subjects will exercise for 15 minutes, during which time the treadmill will be adjusted to the subjects comfort and to reach the desired minute ventilation. They will then rest for 15 minutes, followed by another 15 minute exercise period to confirm the degree of exercise and the treadmill settings. Subjects will be monitored with cardiac telemetry during the treadmill period, and vital signs will be measured after exercise is complete.

A baseline MCC scan, including a transmission scan, will be completed following the treadmill training. (Full description in section f.)

Nasal epithelial lining fluid (ELF) samples will be obtained from both nares. Nasal epithelial cells (NEC) will be obtained from the right nare.

Subjects will be required to return to the research lab 24 hours after the baseline MCC for a 30 minute follow up scan. (V0b).

III. DOSING:
(Visit 1 and 4)
At Visit 1, subjects will be randomized to receive either active gamma tocopherol or safflower oil placebo. Visit 1 must occur within one month of the baseline visit. Visit 1 may be combined with Visit 0a.

1. Vitals signs will be measured.
2. Urine pregnancy test will be performed on all women of childbearing potential.
3. Spirometry will be performed.
4. Exhaled NO will be measured.
5. Sputum induction will be performed.
6. Subjects will be given study drug prepared by the Investigational Drug Service and instructed to take the first dose of study drug the evening of the visit, 2 days prior to the planned exposure. The second dose will be taken 12 hours later, in the morning on the day prior to the exposure. Dose three will be taken 24 hours after the first dose. The final dose will be taken on the morning of the exposure, 12 hours after dose 3.

IV. OZONE CHALLENGE (after 4 doses of study drug)
(Visit 2 and 5):
Pre-Exposure

1. Urine pregnancy testing if applicable
2. Vital signs
3. Ozone Symptom questionnaire
4. Exhaled NO measurement
5. Spirometry
6. Subject will undergo brief physical examination prior to exposure

7. Blood samples obtained for phagocytic function, γT,γT metabolite, αT, cytokines, proteins, immune and inflammatory markers, eicosanoids, glutahione levels, PT, aPTT, CBC with differential, CRP, plasma lipids, and other antioxidants, as well as flow cytometry studies; RT-PCR will also be performed for gene expression studies.
8. ELF samples will be collected.
9. Standard telemetry monitoring will commence

Ozone exposure (0.25 ppm)

1. The subject enters the chamber, and proceeds to the treadmill. This is time point 0 for data collection. Exercise starts at the settings determined on the training day.
2. Minute ventilation is measured at 8 minutes into the exercise period, and again at 12 minutes, for 2 minutes each time. For subsequent exercise sessions, minute ventilation will only be measured at the 12 minute mark.
3. The subject will exercise for 15 minutes, followed by 15 minutes of rest. This cycle is repeated for a total of 3 hours, for **6 exercise and 6 rest periods.**
4. Blood pressure will be measured at least once an hour while they are in the chamber.
5. Symptom scoring will be done immediately prior to exiting the chamber.

Post-Exposure

1. Spirometry and vital signs will be measured immediately post exposure.
2. Elf samples will be collected.
3. Spirometry will be collected again at approximately 30-45 minutes post completion of the exposure.
4. One hour after O_3 exposure is completed, the subject begins the MCC scan procedures. Spirometry will only be collected during MCC scanning if the subject has distress.
5. At completion of MCC, vital signs and spirometry will be obtained. This should approximately 6 hours after the beginning of the exposure time point.
6. Post-exposure blood samples will be collected.
7. Sputum induction, with albuterol pre-treatment.
8. Elf samples will be collected.
9. NEC will be collected from the left nare.
10. Subjects will return to the gamma camera for a final 10-minute scan.
11. Discharge home with contact information for a study physician.

V. FOLLOW-UP:

(Visit 3 and 6)

Subjects will arrive in the research lab approximately 24 hours after the start of the exposure. Subjects will undergo:

1. 30 minute follow up MCC scan.
2. Vital sign collection and symptom scoring
3. Blood sample collection
4. Exhaled NO
5. ELF
6. Spirometry

Subjects will be discharged with Ozone Symptom questionnaires to be filled out on each of the 4 days following the Follow-Up Visit, and with contact information for a study physician.

A follow up phone call will be made to the subject approximately 7 days after discharge.

VI. WASHOUT PERIOD

Subject will not take any study treatment for a minimum of 3 weeks and a maximum of 3 months.

VII. CROSSOVER

Subject will repeat steps **II** and **III** with the SO (placebo) if they received yT before crossover, or the subject will receive yT if they received SO before crossover.

VIII. END OF STUDY VISIT:
(Visit 7)

Within 5-10 days of the final challenge dose, each subject will be asked to return for a study discontinuation visit. At that time, the following assessments will be performed: vital sign measurement including O_2 saturation; lung function (FVC and FEV1); CBC, PT and aPTT (if abnormal values at Visit 6) and Ozone Symptom questionnaire. If any of these suggest lingering effects of ozone exposure, medical evaluation as directed by the study physician will be undertaken.

The table below summarizes the procedures at each visit:

Assessment/ Procedure	**V0**	**V0a**	**V0b**	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**
Informed consent	**X**									
Medical History	**X**									
Review history/AE's		**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**
Record Concomitant meds	**X**	**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**
Urine pregnancy test	**X**	**X**		**X**	**X**		**X**	**X**		
Vital signs	**X**	**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**
12 lead ECG	**X**									
Spirometry	**X**	**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**
Physical exam	**X**				**X**			**X**		**X***
Blood draw	**X**				**X***	**X**		**X***	**X**	**X***
Ozone Symptom Questionnaire					**X****	**X**		**X****	**X**	**X**
Sputum Induction	**X**			**X**	**X**		**X**	**X**		
Exhaled NO	**X**	**X**		**X**	**X**	**X**	**X**	**X**	**X**	
ELF		**X**			**X****	**X**		**X****	**X**	
NEC		**X**			**X**			**X**		
Telemetry		**X**			**X**			**X**		
Treadmill training		**X**								
Study drug dispensed				**X**			**X**			
Medication Diary disp.				**X**			**X**			

Lung transmission scan		**X**			**X**			**X**		
MCC		**X**			**X**			**X**		
Follow-up scan			**X**			**X**			**X**	
Ozone challenge					**X**			**X**		

*These assessments are performed twice at this visit – before ozone exposure and after ozone exposure
**These are performed three times at this visit – pre-exposure, immediately post- and 6 hours post- exposure
*** These are done as needed, as determined by the study doctor.

e. <u>Dosage</u>:

1. Gamma Tocopherol:

γT geltabs (and identical matching safflower oil placebo geltabs) will be provided by Andreas M. Papas PhD, President of Callion Health, Inc. and Adjunct Professor, East Tennessee State University, Johnson City, TN.. Subjects will take 2 geltabs (1200 mg/dose) every 12 hours for a total of 4 doses.. Each geltab will be composed of 612 mg γ-tocopherol, 7 mg d-α-tocopherol, 28 mg d-β-tocopherol, and 8 mg d-δ-tocopherol. This γT preparation is currently in use by our group under a separate IND. The geltabs will be taken with meals to maximize bile secretion in order to enhance absorption of the lipid material including tocopherols

The 48 hour supply of study drug will be dispensed from the Investigational Drug Pharmacy to the CEMALB study staff who will provide the drug to the volunteers, Subjects will be instructed to take the first dose in the evening at approximately 8pm, 2 days prior to the planned exposure. They will be instructed to take dose 2 at 8am the next day, at 8pm that same day and at 8am the following day. The 4th dose will be taken at the CEMALB. Subjects will be randomized to either orally administered gamma tocopherol or safflower oil during the initial study period, and will receive the alternative drug during the crossover period.

Subjects will be allocated to the first treatment group using permuted block randomization with a block of size 4**.** Thus after every 5th subject is entered, there will be two subjects in the placebo group for every 2 subjects in the gamma tocopherol group. The randomization list will be prepared by a biostatistician and provided to the pharmacy. Only the pharmacist and the statistician will have access to the randomization schedule. Once a patient is found to be eligible for randomization, the clinician will contact the pharmacist and ask for medication. The pharmacist will provide the medication as the next allocation on the randomization list. If it is necessary to break the blind, the pharmacy will be contacted and will provide the treatment allocation. During the crossover period, the pharmacist will provide the alternative study medication to a subject (i.e. if the subject was randomized to receive gamma tocopherol during the initial treatment period, they will be provided placebo during the crossover period.

This section outlines CMC information on the ozone used as a challenge agent in a dose-ranging clinical study to assess the effect of pre-treatment with a single dose of inhaled fluticasone propionate on lung inflammation following challenge with inhaled ozone and intermittent exercise in healthy volunteers, relative to placebo. This amendment includes details of the description, composition, and manufacturer, method of manufacture, specifications and analytical methods.

2. Ozone (Challenge Agent)

Description

As described in the IND, Ozone is a highly reactive gas that is commonly found as a major component of photochemical smog and acute exposure to ozone is known to cause airway inflammation. For the purpose of this challenge study, exposures to ozone via inhalation are conducted in a 4 x 6 x 3.2 m stainless-steel chamber with continuous reconditioning of the chamber air through high-efficiency particle filters. Ozone is generated by exposure of oxygen to a silent electric arc in an ozone generator. The concentration of the ozone is continuously monitored to ensure that the appropriate concentration is maintained.

Composition

The chamber atmosphere will be maintained by continuous conditioning of the chamber air to ensure a composition of ozone of 0.25 ppm (± 0.0125 ppm) over 3 hours at 22°C ± 1.1°C and 40% RH ± 5% RH.

Manufacturer(s)

The ozone is made in-situ for use in the environmental chamber that is located as follows:

EPA Human Studies Facility
104 Mason Farm Road
Chapel Hill
NC 27599-7315

Method of Manufacture

Ozone is generated by a Fischer, model 502 ozone generator (Meckenheim/Bonn, Germany). The generator uses oxygen which passes through a silent electric discharge to generate ozone. The ozone concentration and output is controlled by varying the flow of oxygen through the generator and by varying the power of the silent electric arc. The ozone is mixed with the air to obtain a desired concentration. The resulting ozone enriched air is then introduced into the environmental chamber to allow exposure via inhalation.

The concentration of ozone is continuously[1] monitored using UV photometric analyzers. The analyzers are connected to the Analyzer Support System comprising of:

(1) Analyzer Function Support System. This provides vacuum and gas shut-off valves, and potentiometers for the mass-flow controllers;.

(2) Analyzer Calibration System. This consists of equipment to provide accurate calibration of the gas analyzer;

(3) Analyzer Alarm System. Comprises computer software which controls alarms (both sound and visual (light) warnings) and gas shut-off valves.

Description of the Manufacturing Process

The ozone delivery system comprises the following components:

Gas supply bottles and manifolds in the bottle house with oxygen cylinders;
Gas delivery panels;
Gas injection system;
Ozone generator;
Deionized water system;
Computers and computer interfaces.

Stainless steel gas plumbing is used to deliver oxygen to the pollutant delivery system on demand. Mass-flow controller precisely control the amount of oxygen that is supplied to the zone generator and subsequently mixed with facility makeup air. To ensure that the flow of oxygen is not interrupted, each gas manifold (equipped with a pressure regulator) will automatically change from one bank of cylinders to another. The line pressure, of approximately 50 psig, is monitored by a computer. Deionized water is used to humidify the air.

f. Observations and Measurements

1. Primary Endpoints: The primary efficacy endpoint (airway neutrophils/mg in the sputum) will be assessed by post-O3/pre-O3 ratio of log sputum PMNS/mg, comparing the ratios during the placebo treatment to the active treatment arms.

2. Safety measurements: Symptom scores, lung function tests, PT and aPTT and platelet counts will be assessed as safety measures. Tocopherols in high doses may rarely have an effect on coagulation.

3. Exploratory Endpoints:

Cytokines and other proteins at baseline and in response to ozone: We will examine sputum and plasma samples for levels of inflammatory cytokines, including (but not limited to) GM-CSF, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, RANTES, eotaxin and TNFα. Also to be examined are metalloproteinases, mast cell tryptase, eosinophil cationic protein and eosinophil derived neurotoxin. CRP will also be measured at baseline and 24hr post-exposure.

***Airway and blood phagocyte function*:** We have observed that inhalation of inflammatory stimuli (including ozone and ozone) is associated with changes in cell surface markers of monocytes, macrophages, and neutrophils, especially CD11b (CR3 receptor, associated with response to complement and egress of cells from the circulation to tissue), CD16 (FcRγI receptor for IgG), CD64 (FcRγIII receptor for IgG), CD45 (pan leukocyte marker), CD80 and CD86, MHC class II molecules, and CD14 (an endotoxin receptor). We have also observed that the ability of monocytes and macrophages to phagocytize opsonized zymosan is blunted following challenge with inhaled inflammatory stimuli [(1)]. Similar observations have been made in asthmatics with increased eosinophilia [(2)]. We will examine the effect of γT supplementation on these important parameters of host defense.

***Microarray analysis*:** We will analyze mRNA isolated from sputum macrophages and granulocytes using DNA microarray technology to assess genes involved in innate immune and oxidant stress responses before and after ozone challenge. We will also analyze protein mediator profiles of these cells using the sputum supernatants. These techniques will allow us to examine if γT supplementation modifies innate immune and oxidant stress responses.

Immune Markers: We will also examine cell surface markers of lymphocyte subsets (CD3, CD4, CD8, CD45), monocytes (CD14), NK cells (CD16/56), B cells (CD19/20) and markers relevant to allergic responses (CD23, FcERI)

Eicosanoids: We will examine sputum and plasma samples for levels of cyclooxygenase pathway products (PGE_2, $PGF_{2\alpha}$) and lipoxygenase pathway products (LTB_4, LTC_4, LTD_4, LTE_4) as γT has observed actions on eicasanoid production.

Plasma lipids: Panel will include cholesterol, LDL, VLDL and HDL and triglyercides.

Markers of inflammatory cell activity: To assess neutrophil activity in the airway, we will assay sputum for meyloperoxidase (MPO) and 5-chlorouracil (a product of the action of HOCL derived from neutrophil MPO and nucleobases of injured cells, and a marker of PMN oxidative burst). To assess for eosinophil activity, we will assay sputum for eosinophil cationic protein (ECP) and 5-Bromouracil (a product of the action of HOBr derived from eosinophil peroxidase and nucleobases of injured cells, and a marker of eosinophil oxidative burst). Reactive nitrogen oxide species (RNOS) such as peroxynitrite are generated by inflammatory cells following stimulation with inflammatory irritants and sputum and plasma samples will be assayed for 5-Nitro-γ-tocopherol, a product of the action of RNOS on γT.

Nasal epithelial lining fluid: We will examine epithelial lining fluid for levels of inflammatory cytokines, including (but not limited to) GM-CSF, IL-1β, IL-4, IL-6, IL-8, IL-13, IL-17, RANTES, eotaxin and TNFα. We will also examine levels of metalloproteinases and eosinophil cationic protein.

Nasal epithelial cells: We will analyze mRNA isolated from nasal epithelial cells using a customized Nanostring immune panel to assess expression levels of genes involved in innate immune and oxidant stress responses before and after ozone challenge. Genes examined will include those implicated in various aspects of epithelial-driven inflammatory responses, such as those associated with epithelial barrier integrity (apical junction complex proteins), epithelial-derived danger signals, pro-inflammatory chemokines/cytokines, PRRs (pattern recognition receptors), downstream targets of PRR signaling, and genes associated with the antioxidant response element.

Other Antioxidants in plasma and sputum samples: These measures will be provided by the Biochemistry Core of the UNC Clinical Nutrition Research Unit and may include reduced and oxidized glutathione, catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase.

4. Hypertonic Saline Induced Sputum procedure:

FEV1 and force vital capacity (FVC) are measured before ozone challenge to determine the post bronchodilator baseline FEV1, and FVC values. The FEV1 values that match a 10% and 20% fall from post bronchodilator baseline are determined. An ultrasonic nebulizer is filled with 15cc of 3% hypertonic saline (inhalation grade for respiratory use only, 3% NaCl) to begin the test. The nebulizer is set to the maximum output setting and turned on. The subject is instructed to latch his/her mouth onto the nebulizer mouthpiece and breath normally (i.e., tidal breaths) for 7 minutes. The saline is nebulized through the mouthpiece in a jet stream and inhaled. The nose is not occluded for this procedure. The subject is encouraged to come off the mouthpiece at any time to cough if a sputum sample from the lower airways (i.e. not from the back of the throat) is ready for expectoration. Prior to expectoration, subjects are asked to blow their nose, rinse their mouth with water, and clear their throat to avoid the inclusion of non-airway fluid samples. The sample is expectorated into a sterile specimen jar and capped. Following the measurement of FEV1 after the first 7 minute inhalation period,

the concentration of saline is increased from 3% to 4%, provided the FEV1 falls by < 10% from the post-bronchodilator value. A volume of 15cc fills the nebulizer well on each occasion. If the FEV1 falls between 10-20% of the post bronchodilator value, the test proceeds but the concentration of saline remains the same If the FEV1 falls by > 20% or if troublesome symptoms occur, the nebulization is discontinued and albuterol is available if necessary to relieve symptoms. Troublesome symptoms included cough, chest tightness, and general discomfort. The same procedure is followed for the final 7 minute inhalation period using 5% hypertonic saline provided the FEV l safety parameters described above have been met. The nebulization is stopped after 21 minutes or earlier if a sputum sample of good quality is obtained (i.e. visible sputum plugs).

5. Spirometry:
Standard methodology conforming to the American Thoracic Society guidelines for measurement of spirometry will be used.

6. Fraction of Exhaled Nitric Oxide
Airway inflammation will be evaluated using the NIOX MINO device, following ATS, 2005 recommendations and the manufacturer's instructions. Since spirometry can potentially impact the nitric oxide (NO) measurement, the FeNO test needs be completed prior to spirometry. In addition, subjects should not eat or drink 1 hour prior to having the FeNO, as this may affect the results.

7. Nasal Epithelial Lining Fluid (ELF): Nasal ELF will be sampled with a small strip of Leukosorb paper inserted gently by means of direct vision into the nasal cavity laterally against the anterior portion of the inferior turbinate. Sterile saline solution is transferred into a mist bottle (the same one used for NL) and subjects are asked to moisten their nasal passages with a single spay into each nostril. A nasal clip will be applied, and the Leukosorb paper will be left for absorption for 2 minutes. Subjects will be required to breathe though the mouth during this time. Thereafter, the Leukosorb strip will be removed and immediately stored at $80^{\circ C}$.

8. Nasal Epithelial Cells (NEC): a non-invasive biopsy procedure will be performed to retrieve a small cluster of cells from each of the nasal cavities. For the nasal biopsy procedure, the subject will be seated comfortably in a straight-backed chair or reclining on an examination table with the head tilted as far back as possible while remaining comfortable. Subjects will be given no medication, sedation, or anesthesia for this procedure. A short, sterile plastic sampling device called a curette will be inserted into one of the nasal cavities and the surface of the nasal cavity will be stroked several times for approximately 5 seconds in order to obtain a small cluster of cells. This maneuver will be repeated on the contralateral nasal cavity using a fresh curette. The nasal biopsy may only be obtained from 1 nostril during a visit.

9. Regional Deposition and Mucociliary Clearance (MCC) procedures:
Prior to the each MCC study a transmission Co57 scan will be performed to define the lung boundaries, to assign regions of interest, and to normalize these regions for lung volume differences. A rectangular phantom containing the radioisotope Co57 (< 25 mCi) will be placed in front (5cm) of the subject sitting with his/her back to the gamma camera for 30 seconds. The transmission scan has been used by us and others to provide a delineation of lung boundaries for assessing regional deposition/clearance of the inhaled radioaerosol. Prior to the transmission scan on each study day we will place 2 spot markers of Americium241 (0.9

microcurie (uCi) each, gamma 66 KeV) on the upper and lower back of each subject during scanning (both Tc99m-SC deposition/retention and Co57 transmission). With dual isotope imaging, these spot markers will allow alignment of images for more accurate determination of regional deposition/retention. These very low radiation sources have been obtained from commercially available home smoke alarms. The placement of these markers will be determined to be outside the lung field during the transmission scan. Their location will be marked in semi-permanent ink for later placement during Tc99m deposition/retention scans. The shielded side of this source will be placed/taped onto the subject’s skin.

Radiolabeled Tc99m-sulfur colloid will be delivered using a modified Pari-LL nebulizer (MMAD 9.5 um). This is a closed delivery system that produces 80 ml/sec air flow, and therefore limits the inspiratory flow rate to this value. While seated in front of a gamma camera subjects will perform single inhalations lasting ~10 seconds each from the delivery system, and will exhale at 500 ml/sec (using feedback from a flow meter in the breathing circuit). Approximately 5-10 of these inhalation maneuvers will be required to deposit an adequate radioisotope dose to the lung. Subjects will be allowed to breathe normally (off the nebulizer) in between each inspiratory maneuver. Each volunteer will practice these maneuvers prior to the actual radioaerosol inhalation to guarantee his/her proficiency. The activity of Tc99m-SC loaded in the nebulizer will be adjusted to provide an estimated 40 uCi deposited in the lung for each MCC scan. A single crystal detector will be placed at the subject’s back during inhalation to monitor dose to the lung. Total inhalation time should be less than 5 minutes in all cases. Immediately following radioisotope inhalation, the subject will gargle and drink water to clear activity that deposited in the mouth into the stomach. The subject will then (within a minute of final inhalation maneuver) be seated in front of a large-field-of-view gamma camera to begin acquiring particle retention images. Serial, 2-minute gamma images will be captured continuously for the first 34 minutes following isotope delivery. Thereafter, 2 consecutive 2-minute images will be obtained at the start of every 10 minute period until 2 hours post isotope inhalation. The subject will also return the following day to obtain a 30 minute scan of 24 hour lung activity.

Whole lung clearance is strongly dependent on the site of particle deposition within the lung. To characterize regional deposition in the lung a transmission scan of the subject’s lungs will be obtained on each MCC study day to outline the area of the whole lung. Central (C) vs. peripheral (P) deposition will be determined by region of interest (ROI) analysis and normalized to C/P for the transmission scan. A whole lung ROI bordering the right lung will be used to determine, by computer analysis, the whole lung retention, as a fraction of the initial counts in the right lung, over the two hour clearance period and at 24 hours. The computer program decay-corrects the Tc99m counts throughout the clearance period. Both C and P lung retention will also be determined to allow comparisons of MCC from a region with a preponderance of large, bronchial airways to a region lacking such airways.

10. Symptom Scores:

Subjects will be asked to rate a variety of possible symptoms during the dosing period, including the following: headache, irritation of the nose stuffy nose/sinus congestion, runny nose, dry/sore throat, pain on deep inspiration, unusual fatigue or tiredness, eye irritation, sneezing, shortness of breath, cough, wheezing/whistling in chest, chest tightness, sweating and other.

The following symptom score assessment will be made:

0 = NONE	(not present)
1 = TRACE/NOTICED	(barely detectable)
2 = MILD/LIGHT	(present, but not annoying)
3 = MODERATE	(present, but somewhat annoying)
4 = SEVERE/HEAVY	(present and very annoying and painful)

The highest possible score will be 60 and the lowest is 0. A mild score for every criteria is 15, moderate is 30 and severe is 60.

11. Venipuncture: Blood samples totaling approximately 50ml will be taken each week for a total of 150ml. An additional volume of 6ml will be taken during each O3 exposure.

Observation Schedule: Prior to any inhalation challenge, subjects will undergo a physical examination of the ears, nose, throat and chest and will have an assessment of vital signs (temperature, pulse, respiratory rate, blood pressure), oxygen saturation, symptom score assessment and undergo spirometry to rule out acute illness prior to challenge. All female volunteers will undergo a urine pregnancy test. The timing for collecting study endpoints in relation to the exposure is that time 0 is when the subject enters the exposure chamber. Induced sputum will be obtained on the screening day and again at six hours after each exposure. Sputum will be analyzed for PMN content, CD14 expression on airway macrophages and monocytes, soluble CD14 levels, cytokine levels in sputum and products of inflammatory cells (eosinophil cationic protein, myeloperoxidase). Blood will be collected for a CBC and differential and other exploratory endpoints when the pre- challenge induced sputum is obtained and 6 hours after challenge.

Any subject who is asked to withhold ICS for the purpose of this study will have baseline asthma symptom scoring done. These subjects will complete scoring each day that they withhold medication. They will be given contact information for a study physician who will be available 24 hours a day. They will have rescue albuterol available. Subjects will be discontinued from the study and returned to prior dosing regimen if they have sustained increase in asthma symptoms. Subjects will be treated with prednisone if needed for an exacerbation.

Post-Challenge Observations Reporting:
All volunteers will be discharged home after each challenge day with contact information for a study physician.

Each volunteer will be given a symptom scoring sheet for each day up to 96 hours (4 days) after challenge. The following instructions will be written on the sheet:

> Please record your symptom score today at noon based on the scale above. Please call the study coordinator or physician for the following reasons:
> 1. If your total score is >6 plus your baseline score.
> 2. If any of your individual symptoms equals 4.
> 3. If you are concerned about symptoms regardless of the score you assign them.

Each sheet will include the name and phone number of the study MD. Sample home symptom scoring sheets and instructions are included with this protocol.

Study discontinuation visit (Visit 7):
Within 5-10 days of the final challenge dose, each subject will be asked to return for a study discontinuation visit. At that time temperature, pulse, systolic and diastolic BP, respiratory rate, FVC and FEV1 and SpO2 (oxygen saturation), CBC, PT and aPTT and symptoms scores will be assessed and, if abnormal, medical evaluation as directed by the study physician will be undertaken.

Definition of an unanticipated problem, or UP, (previously referred to adverse event AE) and Serious Adverse Event (SAE):
An unanticipated problem for a given volunteer will be defined as failure of any of the safety criteria outlined in section "d." Additionally, minor upper respiratory tract infections occurring within 96 hours of the exposure will be considered adverse events. Other, non-specified clinical illnesses, which occur within 96 hours of each challenge, will also be reported as an AE. Any decrease in lung function or increase in symptom score, as outlined in section "d" will be considered an adverse event. Failure of a total symptom score to return to no greater than 6 above baseline within 96 hours will be considered an adverse event. Any symptoms that induce a volunteer to seek medical attention from any provider within 96 hours of challenge will be considered an adverse event.

A significant adverse event will be defined as any event that requires hospitalization or results in, life threatening illness or injury, permanent (or likely to be permanent) illness or injury, or death if these events occur within 96 hours of challenge (or if the clinical scenario leading up to hospitalization, illness, injury or death begins within 96 hours of a challenge).

g. Monitoring and risk minimization

Risks to subjects: At the doses proposed in this study, the most significant predictable risk to subjects as a result of ozone inhalation is development of airway inflammation which would cause shortness of breath, cough or wheeze. Ozone is known to cause a temporary inhibition in the ability to take a deep breath – this is transient and typically declines within 2 hours of completion of exposure, though it may take up to 72 hours for complete resolution. At high doses, tocopherols can increase coagulability, though we have not observed this in other studies using gamma tocopherol. Nausea and upset stomach have been reported, subjects are asked to take the supplement with food. Sputum induction may induce bronchospasm. Venipuncture carries a risk for hematoma. There is a slight risk of sneezing with ELF collection, subjects who may not be able to tolerate the paper will not be asked to continue with this measurement. Nasal epithelial biopsy is a transiently irritating procedure, and it presents a rare risk of nose bleed.

Measures to minimize risk: Only volunteers who have normal lung function will be recruited for this project. Further, subjects will be deferred for challenge until 4 weeks after complete resolution of each of the following acute illnesses: viral respiratory tract infection, pneumonia or bronchitis requiring antibiotic therapy (must be off antibiotics and well for 2 weeks after the last dose of antibiotics, 4 weeks in the case of azithromycin due to its prolonged half-life), or acute illness resulting in fever. Also, unspecified illnesses, which in the judgment of the investigator increase the risks associated with ozone inhalation challenge, will be a basis for exclusion. As outlined above in section "d", all subjects will need to fulfill objective lung function and symptom criteria prior to initiating a specific challenge study. Subjects will be questioned about their previous radiation exposure history. Subjects whose exposure history

within the past twelve months would cause them to exceed their annual limits will be excluded. Upon being accepted into the study each subject will be advised to avoid radiation doses of a comparable magnitude within the next 12 months unless there is a diagnostic or therapeutic necessity. Female volunteers will be asked to use effective birth control and will provide a urine sample to test for pregnancy on study days. If the test is positive or the subject has reason to believe she may be pregnant, she will be dismissed from the study. Nasal samples will not be collected in subjects who have had recent nasal surgery.

Risks from MCC measures

The radiation doses for up to 120 μCi of inhaled Tc99m-SC (3exposures of 40 μCi over the three month period) to various organs (other than the lungs) are listed below:

ORGAN DOSE (mRem)
Kidneys 12
Bladder Wall 9
Stomach Wall 30
Thyroid(unblocked) 18
Testes 1.2
Ovaries 5.4
Red Bone Marrow 2.4
Upper Large Intestine 15
Lower Large Intestine 1
Whole Body 1.8

These doses were computed assuming an instantaneous uptake of free TcO4(99m) from the systemic circulation. This simulates the situation where all of the label breaks free from sulfur colloid and is transported instantaneously to the systemic circulation. These doses represent extreme cases that rarely, if ever, occur in practice. Previous studies show no Tc99m activity in the bloodstream of dogs over a 3 hour period after depositing Tc99m-sulfur colloid on the airway surfaces of dogs. TcO4(99m) is secreted by the gastrointestinal tract and there is minimal reabsorption. Thus, if all the label were to break free from the sulfur colloid, the stomach, large intestine, thyroid and kidney which see a large blood flow would receive the highest exposure from TcO4(99m). Since the sulfur colloid particles are not water soluble they are not cleared by dissolution.

For our inhalation conditions 30-60% of the inhaled particles will likely clear by mucociliary clearance. Particles that clear by this mechanism will be swallowed and eliminated by the GI tract. Most of the remaining deposited particles will be in alveoli or small airways and clear by much slower processes with a half-time of approximately 60 days. Considering the short physical half-life of Tc99m one need not consider the radiation doses to organs, other than the lungs and GI tract, from Tc99m bound to sulfur colloid. There is potential for enhanced radiation doses to large airway epithelia. The local radiation dose depends on the pattern of particle deposition and on the rate of mucociliary clearance. Using published data and assuming a mucus transport rate in the trachea of 1.0 mm/min (representing the lowest limit measured in normal humans), the highest radiation dose delivered to the trachea will be 0.8 Rems for a single 40uCi exposure. The radiation dose will decline towards the more peripheral portions of the lung reaching 0.016 Rems in the parenchyma or alveolar regions. The unweighted average dose to all tissue compartments will be 0.32 Rems (for a single 40uCi

exposure). These computed doses represent the upper limits since normal tracheal mucus velocity is approximately 5 mm/minute.

Transmission scans will also be obtained to identify non-ventilated lung regions.
These scans will be used to outline/define the whole lung regions for region-of-interest analysis of the Tc99m-SC radioaerosol scans on each study day. For the lung transmission scan procedure, a planar, solid sheet containing the radioisotope Co57 (< 25 mCi) will be placed in front (5cm from body) of the patient sitting with his/her back to the gamma camera while a 30 sec gamma camera scan is acquired. In this case the dose from the Co57 exposure is from an external source (i.e. not inhaled) and is similar over all portions of the lung tissue (i.e. similar to a chest X-ray). The dose to the surface of the chest and the lungs (assuming no chest wall attenuation) under these conditions is 7.5 mRad for a single scan, a relatively small dose compared to the inhaled radioaerosol. As the Co57 source decays over time (270-day half-life) we will increase the time for the transmission scan accordingly to achieve comparable scans of the lung while not exceeding the 7.5 mRad dose per scan.

The transmission scan has been used by us and others to provide a delineation of lung boundaries for assessing regional deposition/clearance of the inhaled radioaerosol. The absorbed radiation doses from the low activity (<1uCi) /energy (66Kev) Americium241 sources, placed on the subjects backs for alignment of images, is insignificant relative to the Tc99m and Cobalt57 doses associated with these experiments.

The maximum doses that we have computed for the lungs (combined three Tc99m and three Co57 transmission), 2.4 Rem local dose and 0.98 Rem average for whole lung, are well under the maximum single dose of 5 Rems for a normal adult volunteer as set forth in Federal regulations, and well under the 15 Rem maximum for the annual dose commitment. The doses to all other organs and the whole body are all far less than these maximum doses. **The total effective dose equivalent (based on the unweighted average to the whole lung of 983 mRem) is 133 mRem**. In considering these dose estimates and comparing it with other nuclear medicine procedures, it should be emphasized that we have gone to considerable lengths in calculating local tissue doses. Most often average doses to organs are used which would be considerably less than any local doses.

While the radiation exposure associated with our measures of MCC represents a potential risk to the human volunteers in this study, the risk is very low. The effective dose for the threemeasures of MCC (including Co57 transmission scans) is approximately 133 mRem. This is less than the natural environmental radiation that adults receive every year, which in Chapel Hill is about 300 mRems. The risk from the radiation dose received from this procedure is too small to be detected. In addition, the dose of Tc99m- sulfur colloid to be deposited in the lung for each measure of MCC is 10% of that recommended as the adult oral dose for diagnosing pulmonary aspiration by gamma scintigraphy (CIS-US,Inc).

A physician familiar with the protocol will be available for all challenge procedures. Emergency treatment with albuterol, oxygen, and oral corticosteroids will be available to those patients who require such therapy. The human exposure laboratory is also equipped with an emergency “crash cart” with standard emergency medications, IV fluids and a defibrillator in the unlikely event of a medical emergency for any challenge or exposure study.

All subjects will be will be monitored as outlined in previous sections and will return for assessment 24 hours after the challenge. All subjects will be provided with a contact telephone number for access to a study physician who is on call 24 hours/day.

Reporting of AEs and SAEs

All SAEs will be reported to the CBER of the FDA as well as to the UNC Biomedical Institutional Review Board (IRB) within 24 hours of recognition of the event. Adverse events will be reported to both the FDA and UNC IRB on an annual basis, or when the protocol is completed. If criteria for suspension of the protocol are met, then the FDA and UNC IRB will be notified within 24 hours.

All subjects with a non-fatal SAE will be evaluated medically by a study physician, in concert with their own physician as appropriate. Likewise all subjects with an unexpected problem related to the study will be evaluated by a study physician, and if needed the subject will be examined by the study physician. All assessments will include the same lung function and temperature assessments outlined for challenge observation. Other assessments will be undertaken as needed. Any unspecified event, which in the judgment of the PI of the study, constitutes an unusual, unexpected or prolonged event (greater than 96 hours) event will be reported to both the FDA and the UNC IRB.

CERTIFICATE OF ANALYSIS

No. 60-1399

Messrs.

17 Jul 2013

タマ生化学株式会社
TAMA BIOCHEMICAL CO.,LTD.
1-23-3, Nishishinjuku,
Shinjuku-ku, Tokyo, Japan

TamaE-G80

(*d*-gamma Tocopherol 80%)

Lot Number : 307111

Tests	Specifications	Results
Description	A light yellow to reddish brown, clear and viscous liquid, having a faint characteristic odor	Good
Identification	Red to orange color development	Passed
Acidity	Not more than 1.0 ml/g	0.1 ml/g
Heavy Metals (as Pb)	Not more than 10 ppm	Within Limit
Arsenic	Not more than 1 ppm	Within Limit
Transmittance	Not less than 20.0 % (550nm)	73.7 %
d-gamma Tocopherol	Not less than 80.0 %	90.0 %
Total Tocopherols	Not less than 90.0 %	94.7 %
Benzo(a)pyrene	Not more than 1.0 ppb	0.1 ppb
Aerobic Plate Count	Not more than 1000 cfu/g	Within Limit
Coliforms	Negative	Passed
Residual solvents	USP <467> only class 2 solvents	Within Limit
Evaluation		Passed

Manufacturing Date : 11 Jul 2013
Expiry Date : 10 Jul 2016
Lead (random test) Not more than 2 ppm (FCC)

Remarks : This lot is not derived in whole or part from animals sources. Then it has no risk of BSE. This lot is not treated with a radiation. This lot does not contain Melamine, Ethylene Oxide and any allergenes.

T. Noguchi

NOGUCHI Takahiro
Manager of Quality Control Div. of KOFU Plant
ISO9001 Certified JQA-QM6932