REPORT

Study Title:	Effects of inhaled calcium glycerophosphate on Rhinitis Responses in Ragweed Sensitized Dogs
LRRI Protocol Number:	FY09-133
Test Facility:	Lovelace Respiratory Research Institute (LRRI) 2425 Ridgecrest Drive SE Albuquerque, NM 87108 Location of Laboratory: Bldg 9217, Area Y Kirtland Air Force Base Albuquerque, NM 87115
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Alternate Sponsor Representative:	Charles Bove, Director, Clinical Studies

3/09/2010
Sponsor Representative Date

3/3/2010
LRRI Study Director Date

Alan B. Khorram
CEO

TABLE OF CONTENTS Page No.

1.0 EXECUTIVE SUMMARY

The goal of the study was to determine whether repeated inhalation and intranasal instillation of calcium glycerophosphate attenuates ragweed induced nasal congestion and has an effect on nasal inflammation. Pretreatment with the compound at the dose achieved (1.6 mg inhaled and 30 mg intranasally instilled) significantly attenuated nasal congestion and had some minor effects on the early mediators measured in nasal lavage fluid collected for up to 60 minutes after nasal ragweed challenge. There was a general trend towards a reduction in some mediators (histamine, leukotrienes and prostaglandin D_2) but none reached statistical significance. When PGE_2 levels are normalized to baseline levels it suggests that there is a greater reduction following RW challenge in conjunction with calcium glycerophosphate treatment. This may suggest that PGE_2 as a potential underlying mechanism for the effects of calcium glycerophosphate. Compound pretreatment did reduce eosinophil numbers at day 2 post ragweed challenge but this did not reach statistical significance. Treatment with calcium glycerophosphate was well tolerated by the animals and no visible adverse clinical signs were observed or any significant effects on blood chemistry parameters. Overall, the response seen following calcium glycerophosphate is similar to responses we have previously observed with an α-adrenergic agonist, pseudoephederine, a Histamine H1 antagonist, Chlorpheniramine, and Montelukast in this model.

2.0 REGULATORY COMPLIANCE

This study was conducted in the spirit of U.S. FDA 21 CFR Part 58 (Good Laboratory Practice for Nonclinical Laboratory Studies), but not all study aspects were within strict compliance.

This study complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR Parts 1, 2, and 3) and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

3.0 KEY STUDY PERSONNEL

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4.0 TEST ARTICLES

One test article at one dose was screened in the efficacy experiment. The test article was characterized by the manufacturer. Prior to the experiments the compound was tested in the aerosol exposure system and a maximum dose of 20 mg/ml could be used for the nebulization system (see Appendix 1).

4.1 Test Article

Identity:	calcium glycerophosphate Increased solubility at a lower temperature and citric acid increases its solubility in water DO NOT USE CITRIC ACID – RECORD Ph OF COMPOUND SOLUTION AT ADMINISTRATION TO BEAGLE DOGS
Description:	The raw material is in granulate (powder) form, 100 mesh (less than 150 microns)
Supplier:	AKPharma
Manufacturer:	Astha Laboratories, Heyderabad, India
Storage Conditions:	Room temperature, desiccated, and protected from light
Handling Precautions:	Wear lab coat, gloves, eye protection, and a surgical mask or respiratory protection when handling the neat test article.
Handling Precautions:	Wear lab coat, gloves, eye protection, and a surgical mask or respiratory protection when handling the neat test article.

4.2 Vehicle

Identity:	Sterile water.
Description:	Clear liquid
Supplier:	Phoenix Pharmaceuticals, Inc
Manufacturer:	Phoenix Pharmaceuticals, Inc
Storage Conditions:	Room temperature
Handling Precautions:	Wear lab coat, gloves, eye protection

5.0 TEST SYSTEM

Species/Breed:	Canine/Beagle
Age of Animals at Study Start:	5 dogs Range = 3.25. to 5.25 yrs at start of the study
Body Weight Range at Study Start:	10.0 to 12.5 kg at start of the study
Number on Study/Sex:	5 (1 males and 4 females)
Source:	LRRI Beagle colony
Identification:	1656 A (male) 1668 V (female) 1674 S (female) 1677 U (female) 1678 S (female)

6.0 ANIMAL HUSBANDRY

Housing:	Indoor-outdoor kennel runs
Quarantine Period:	Not applicable
Feed:	2025 Teklad Global 25% Protein Dog Diet (Harlan Teklad, Madison, WI), once daily. The animals were fasted overnight and were fed approximately 3 hours after dose administration. Any remaining food was removed after approximately 4 hours. Note: on days when procedures were performed dogs were not be fed until after all procedures requiring anesthesia were completed.
Water:	Municipal water, unlimited access. Water is analyzed at least annually by LRRI for heavy metals, chlorinated hydrocarbons, organophosphates, nitrates, nitrites, standard plate count, total trihalomethanes, and dissolved minerals. The Study Director reviewed the water analysis documentation.
Environmental Conditions:	The targeted indoor conditions for temperature and photoperiod were as follows: Temperature: 18–29 °C Relative Humidity: 30-70% Light Cycle: 12-h No excursions.
Morbidity and Mortality:	Animals were observed twice daily for any adverse health conditions. No animals exhibited any adverse health

conditions.

Health Status: Only visually healthy animals were used on the study. A laboratory animal veterinarian or their designee visually examined the animals before assignment to study.

7.0 EXPERIMENTAL DESIGN

Beagle dogs immunized with ragweed (RW) as puppies develop allergic immune responses. These allergic dogs show elevated total and specific serum IgE and increased numbers of eosinophils in their blood and lungs, as well as an increase in airway resistance and a decrease in dynamic compliance after a challenge with RW by inhalation or local instillation in defined lung lobes. Additionally, these dogs develop increased nasal congestion and inflammation following RW challenge in the nose. Dogs (n=5) with preexisiting nasal and airway allergic responses were used in the following study.

Dogs served as their own control and initially were treated with vehicle (sterile water) and then rested for 5 weeks prior to receiving the test compound. The target dose of calcium glycerophosphate was ~ 30 mg/dog twice daily. The maximum concentration of the compound used for inhalation and nasal instillation was 20 mg/ml due to solubility issues. Based on the aerosol testing (Appendix 8) only 1.6 mg/dog could be delivered by inhalation during a 15 minute exposure (20 mg/ml solution). In addition, each dog received twice daily an intranasal instillation of 30 mg (2x 375 µl of 20 mg/ml in each nostril) given within 30 minutes after the end of the inhalation exposures.
Dogs were treated with vehicle or compound beginning 3 days prior to nasal challenge with ragweed and treatment was continued until the last day of measurements (day 0 – 5) by twice daily inhalation and intranasal delivery (see Appendix 1).

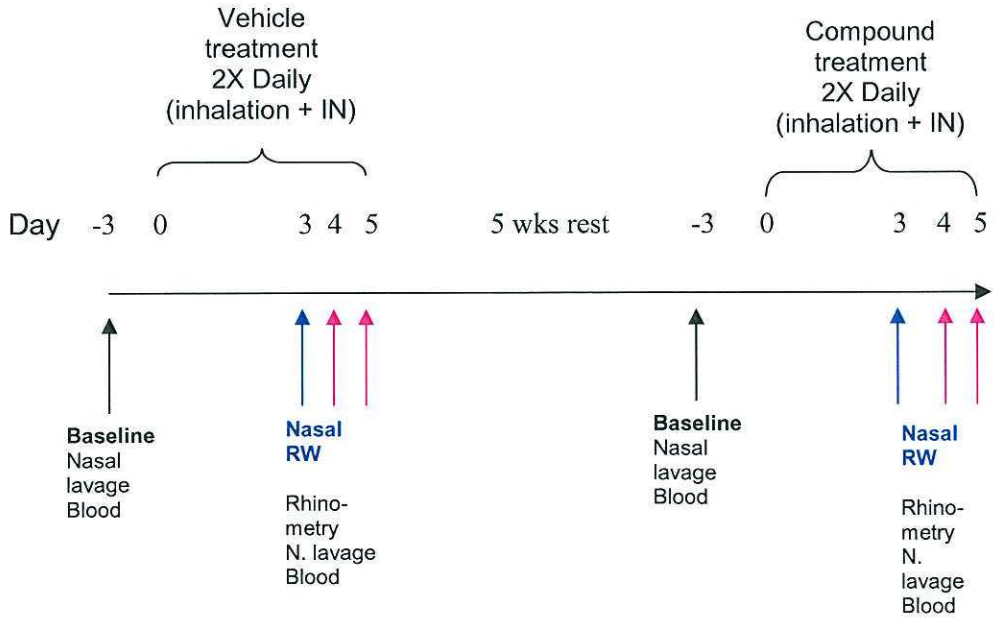

Figure 1. Summary of the study design for characterizing the effects of compound on rhinitis symptoms in ragweed sensitized Beagle dogs.

7.1 Dose preparation and administration

Vehicle (water) was given twice daily by inhalation (15 minutes) followed by intranasal instillation of water (2 x 250 µl in each nostril). The test compound calcium glycerophosphate was formulated in sterile water at a dose of 20 mg/ml und given by inhalation (deposition 1.6 mg). The Sponsor provided the test-article and the compound solution was prepared in the morning of each treatment day. Vehicle and test-article were administered by inhalation and intranasal instillation twice a day.

Treatment occurred on Days 0 to 5 (only AM on Day 5). Vehicle and compound were delivered in the morning (8:15 – 9:10 am) and the afternoon (2:00 – 3:30 pm) as shown in Appendix 1.

On days when acoustic rhinometry procedures were performed (Days 0), the morning dosing occurred such that the procedures were performed within 1 – 6 h after dosing.

The pH of the compound (as administered for nasal inhalation) was recorded prior to administration. As identified in the aerosol exposure the average pH of the nebulizer solution was 8.85.

8.0 OBSERVATIONS AND MEASUREMENTS

8.1 Blood collections

Blood was collected by placing a venous (cephalic or saphenous) catheter in the leg prior to anesthesia or by venipuncture (jugular) using a syringe or needle-vacutainer. On the study Days -3 (baseline), 3, and 5 two 2 to 3 ml blood samples were collected for vehicle and compound experiment prior to exposures and intranasal instillation for chemistry and CBC analysis (Appendix 6).

8.2 Rhinitis Assessments

- Nasal cavity geometry (AcR) pre/post antigen challenge in both nares
  1. time 0 (just prior to RW instillation)
  2. at 15, 30, 45, 60, 75, 90 min, 24h, 48h post RW challenge
- Nasal lavage of both nares at baseline (Day -3), and at 24 (Day 4) and 48h (Day 5) post RW instillation and only one side (right nare) at 15, 30, 45 and 60 min post challenge (Day 3)
  1. Release of histamine, leukotrienes and prostaglandins in nasal lavage fluid (baseline [prior to compound or RW treatment/challenge], 15, 30, 45, 60 min and 24h post RW instillation
  2. Nasal lavage cells from the baseline (Day -3), and 24 (Day 4) and 48 (Day 5) hour lavage were used to determine inflammatory cell differentials
  3. Excess nasal lavage fluid was frozen for analysis of cytokines (not part of this report)
- Cardiovascular readouts (heart rate, O_2 saturation, body temperature) throughout nasal congestion measurement.

8.2.1 Measurement of Nasal Cavity Geometry by Acoustic Rhinometry – Rhinitis Assessment

Acoustic rhinometery was measured on experimental Days 3, 4, and 5. Nasal cavity volume was measured in anesthetized (see 9.2.2 below) dogs using an Eccovision Acoustic Rhinometry System (Hood Laboratories, Inc., Pembroke, MA). Briefly, a wave tube containing a spark sound generator was connected with the nasal cavity using a plastic nose piece. Based on nasal cast impressions and X-ray measurements from the dog nasal cavity, a distance from the nostril opening into the nasal cavity of 10 cm was used for all experiments. Acoustic reflections were converted to area-distance function curves and used to determine nasal cavity volume. Heart rate and O_2 saturation were measured throughout the experiment. Body temperature was checked occasionally. To avoid a rapid drop in body temperature associated with general anesthesia dogs were placed on a water circulating heating pad during the experiment if necessary. Nasal cavity volume was measured before and at different time points after nasal RW challenge (see 9.2.3 below; both nares; 0, 15, 30, 45, 60, 75, and 90 minutes, 24h, 48h post RW challenge). Nasal lavages (see 9.2.4 below) were performed at specific time points before and after RW challenge in both nares at baseline (Day -3), and at 24 (Day 4) and 48h (Day 5) post RW challenge and only one side at 15, 30, 45, and 60 min post challenge (Day 3).

8.2.2 Anesthesia – rhinitis assessment

Dogs were anesthetized with isoflurane (5% induction; 1 to 1.5% maintenance). Briefly, custom made face masks constructed out of rubber material were placed over the muzzles of the dogs for the induction of anesthesia to avoid isoflurane entering the nasal passages. The face masks had a hole cut in the end to allow a brass tubing to protrude out approximately 1 to 2 cm while sealing around its outside. The face masks also occluded the nares of the dogs, thus, assuring mouth-only inhalation of the anesthetic. After inducing anesthesia, an endotracheal tube was placed in the trachea and anesthesia was maintained with isoflurane throughout the experiment. Dogs were placed in a supine position for the nasal congestion measurements and in a prone position with a slightly tilted head for the nasal lavage procedure.

8.2.3 Nasal ragweed challenge by instillation

Ragweed extract (RW short, *Ambrosia artemisifolia*, Greer, Lenoir, NC; 6 mg/ml in 0.25 ml PBS) was instilled in both nares using an Accuspray device (Becton Dickinson).

8.2.4 Nasal lavage

While under anesthesia a flexible plastic catheter was inserted several centimeters into the dog's nare. The nare was washed with a phosphate buffered saline solution (PBS; 3 x 5 ml for collection of cells [baseline, 24 and 48h post RW challenge]; 1 x 5 ml for collection of fluid for mediators [15, 30, 45, and 60 minutes post RW challenge]).

8.3 Pathologic Analyses

Total nasal cells were determined using an automatic cell counter. Cells were spun onto slides by cytocentrifugation and stained with a modified Wright-Giemsa stain. At least four hundred inflammatory cells (or less if applicable) were counted and the percentage of specific cell types determined for each animal (slides in duplicates, 200 cells per slide). The first lavage fluid sample (after centrifugation) was frozen separately for mediator analysis. Mediator analysis for histamine, leukotrienes, and prostaglandins were performed according to the kit manufacturer instructions (Immunotech – Beckman Coulter Company #IM2015, Neogen Corporation #406410, Cayman Chemical Company: PGE2 - #514010, PGD2 - #512011, respectively).

8.4 Daily Observations

Animals were examined twice per day (morning and afternoon) on each day of the study. Examination was oriented toward identifying the onset and progression of any abnormal clinical signs. No adverse health effects were found during the duration of the experiments.

8.5 Body Weights

All animals were weighed on study Days -3, 3 and 5 only for compound experiment (Table 1).

Table 1: Body weight (kg) for all dogs during vehicle and compound treatment.

	Vehicle			Calcium glycerophosphate		
	Day -3	Day 3	Day 5	Day -3	Day 3	Day 5
1656 A	10.5	10.8	NA	10.8	10.4	10.4
1668 V	10.25	10.0	NA	10.7	10.6	10.55
1674 S	12.5	12.7	NA	13.0	13.3	13.2
1677 U	10.5	10.3	NA	10.4	10.3	10.4
1678 S	10.0	9.9	NA	9.8	9.9	9.8

8.6 Statistical Analysis

Changes in nasal cavity volume or mediator levels were assessed by two way analysis of variance (ANOVA) with Bonferroni post-test. All other statistical comparisons were made using ANOVA with Dunnett's multiple comparison test or paired two tailed t-test if appropriate. A value of $p<0.05$ was considered statistically significant.

9.0 RESULTS

A total of 5 ragweed-sensitized dogs were used in this experiment. All dogs received vehicle first and after a 5 week recovery period they were treated with calcium glycerophosphate by inhalation followed by intranasal instillation. Due to solubility of the compound only about 1.6 mg were deposited in the lung by inhalation and a total of 30 mg were intranasally instilled using a BD accuspray device twice a day for a total of 5 days.

The results for all variables (change in nasal cavity volume and mediators in nasal lavage fluid) are shown over time as a comparison between treatment group and vehicle before and up to 24 or 48 hours after treatment, if. The area under the curve (AUC) for the change in nasal cavity volume from baseline (0 to 90 minutes post ragweed) and the change in mediator levels over time (0 to 60 minutes post ragweed) was calculated for each treatment. An increase in area under the curve for compound treatment compared to vehicle indicates an increase in nasal cavity volume and therefore a decrease in nasal congestion (Figure 2). In contrast, a decrease in AUC for the mediator levels (e.g. histamine, leukotrienes, prostaglandins) indicates an attenuation of these mediators due to treatment (Figures 5, 7, 9, 11). An area under the curve of 150 is the equivalent of a 100% increase in nasal cavity volume and no change in AUC (same as vehicle) indicates no change in nasal cavity volume (=0%). The AUC is always shown as an average including standard error for each treatment group and as changes for each individual dog between vehicle and compound treatment.

Repeated treatment (BID) with calcium glycerophosphate by inhalation (1.6 mg depositon) and intranasal instillation (30 mg) starting three days prior to nasal ragweed challenge significantly increased nasal cavity volume and therefore attenuated nasal congestion compared to vehicle treatment (Two Way Anova, $p<0.0001$). The attenuation was statistically significant at all time points from 15 to 90 minutes post ragweed ($p<0.05$, Bonferroni posttest).

The histamine, leukotriene and prostaglandin D_2 and E_2 levels in nasal lavage fluid were partially altered after treatment with the compound compared to vehicle treatment. In general, the levels of histamine, leukotriene and prostaglandin D_2 in nasal lavage fluid increased over time compared to baseline levels after vehicle treatment but no statistical significance was reached between vehicle and compound treatment (One Way Anova followed by Dunnett's posttest). Levels of PGE_2 were elevated in the dogs prior to calcium glycerophosphate treatment (e.g. T_0 [baseline] levels) compared to the start of vehicle treatment-challenge leg. Levels of PGE_2 decreased following ragweed challenge during the calcium glycerophosphate treatment leg (Figure 10). When normalized to baseline levels of PGE_2 there was an overall effect of treatment with calcium glycerophosphate (e.g. reduced levels with respect to baseline) but there was no significant effect at any specific time point (Two-way ANOVA with Bonferroni post test; Figure 11).

Total cell numbers includes inflammatory cells and epithelial cells (Figure 12). Epithelial cells and all inflammatory cells are shown as percent of cells counted (Figures 13 to 17, panel A) and as number of cells based on total number of cells collected (Figures 13 to 17, panel B). In this study the assessment of the number of inflammatory cells in the nasal lavage showed that pretreatment with calcium glycerophosphate did tend to reduce the lung inflammatory cells compared to vehicle treatment but none of the endpoints reached statistical significance. In general, RW challenge resulted in an increase in eosinophils on Day 1 and Day 2 post challenge. Levels of macrophages following vehicle treatment were not detectable and were at negligible levels following calcium glycerophosphate treatment.

Experimental and treatment times for vehicle and calcium glycerophosphate experiments are shown in Appendix 1. Raw data for nasal cavity volume are summarized in Appendix 2 and mediator levels in Appendix 3. The calculated values for the area under the curve for nasal cavity volume and all mediator levels are listed in Appendix 4. Appendix 5 summarizes total cell numbers and cell differentials for all inflammatory cells measured in nasal lavage fluid before and after vehicle and compound treatment. Results for blood chemistry and hematology for individual dogs are listed in Appendix 6. The aerosol development report is attached as Appendix 7

9.1 Nasal congestion

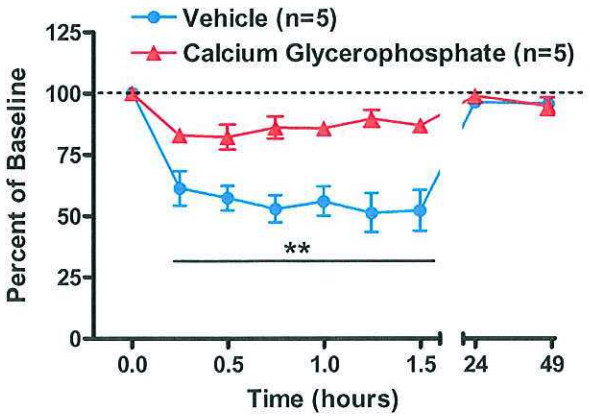

Figure 2: Percent of nasal cavity volume from baseline (time 0) measured before and after intranasal ragweed challenge. Nasal congestion was measured three days after first inhalation and IN treatment with vehicle or CGP (1.5 mg inhale + 30 mg IN). Dogs served as their own control and vehicle experiment was performed about 5 weeks prior to compound treatment. Data are expressed as mean±sem (n=5). Nasal congestion was significantly attenuated compared to vehicle treatment determined by Two Way Anova (p=<0.0001) followed by Bonferroni posttest at individual time points (** p<0.01).

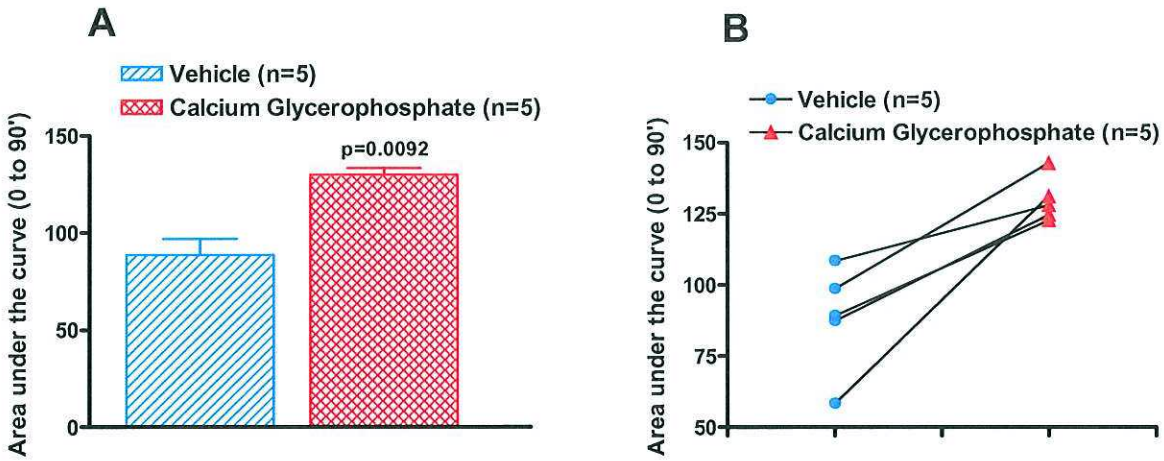

Figure 3: The area under the curve of the change in nasal cavity volume between 0 and 90 min as shown in Figure 2 expressed as mean±sem (A, n=5) and as scatter graph for individual dogs (B) was significantly increased after CGP treatment compared to vehicle control determined by Paired t-test (p=0.0092) indicating an attenuation of the nasal congestion induced by ragweed instillation.

9.2 Histamine in nasal lavage fluid

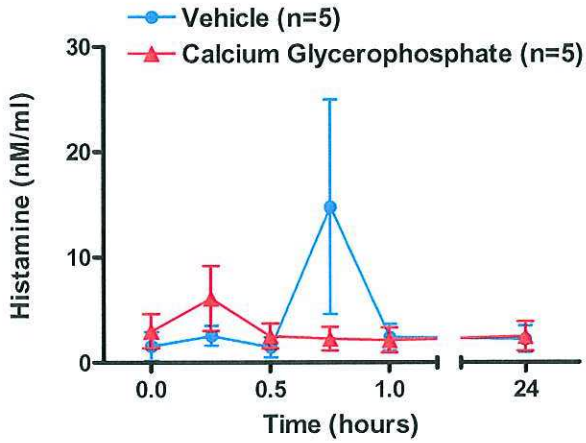

Figure 4: Histamine levels in nasal lavage fluid measured before and after intranasal ragweed challenge done three days after first treatment with vehicle or compound. Data are expressed as mean±sem (n=5). T_0 indicates baseline sample collected the day before the initiation of either vehicle or calcium glycerophosphate treatment. Dogs served as their own control and vehicle treatment was compared to compound treatment. Histamine levels were not statistically significant different between both treatment groups.

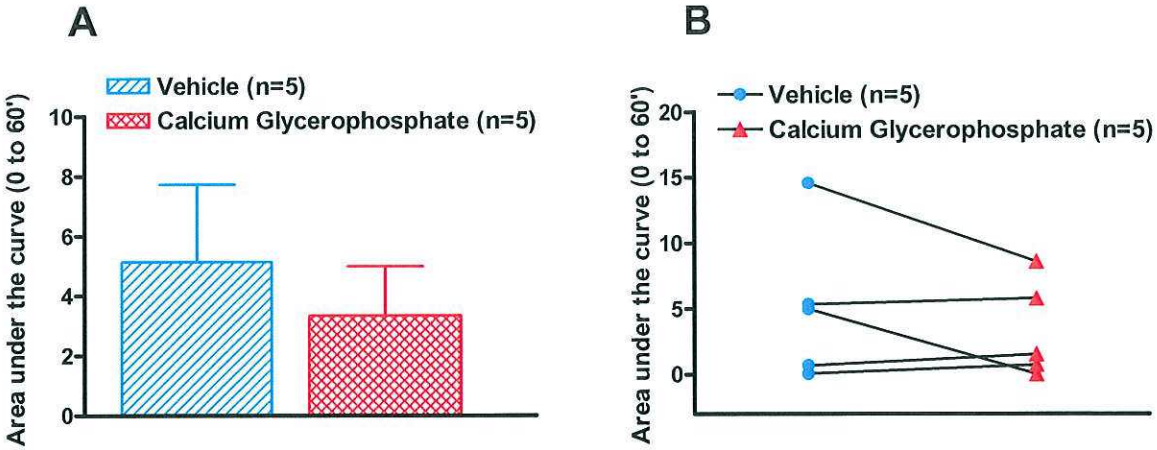

Figure 5: The area under the curve was calculated for Histamine levels between 0 and 60 min (Figure 4) for vehicle and compound treatment shown as mean±sem (A, n=5) and individual data points in a scatter graph shown as change from vehicle to compound treatment (B). Histamine levels decreased only in two of the 5 dogs and no statistical significance was reached.

9.3 Leukotriene $C_4/D_4/E_4$ in nasal lavage fluid

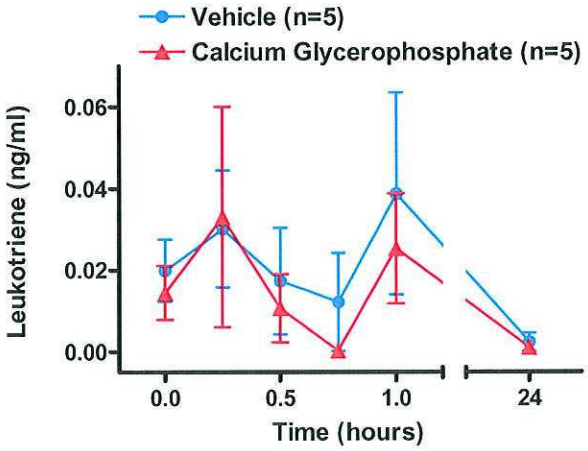

Figure 6: Leukotriene levels in nasal lavage fluid measured before and after intranasal ragweed challenge done three days after first treatment with vehicle or compound. Data are expressed as mean±sem (n=5). T_0 indicates baseline sample collected the day before the initiation of either vehicle or calcium glycerophosphate treatment. Dogs served as their own control and vehicle treatment was compared to compound treatment. Leukotriene levels were not statistically significant different between both treatment groups.

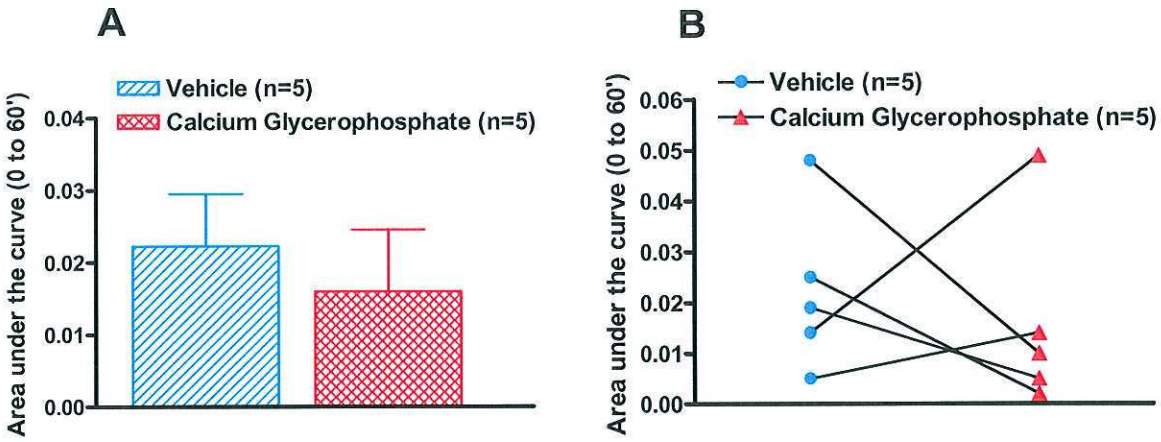

Figure 7: The area under the curve was calculated for Leukotriene levels between 0 and 60 min (Figure 6) for vehicle and compound treatment shown as mean±sem (A, n=5) and individual data points in a scatter graph shown as change from vehicle to compound treatment (B). Leukotriene levels decreased in three dogs and increased in two dogs and no statistical significance was reached.

9.4 Prostaglandin D_2 in nasal lavage fluid

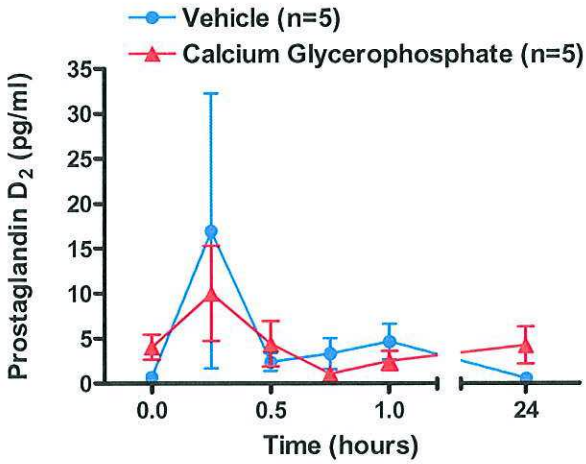

Figure 8: Prostaglandin D_2 levels in nasal lavage fluid measured before and after intranasal ragweed challenge done three days after first treatment with vehicle or compound. Data are expressed as mean±sem (n=5). T_0 indicates baseline sample collected the day before the initiation of either vehicle or calcium glycerophosphate treatment. Dogs served as their own control and vehicle treatment was compared to compound treatment. Prostaglandin D_2 levels were not statistically significant different between both treatment groups.

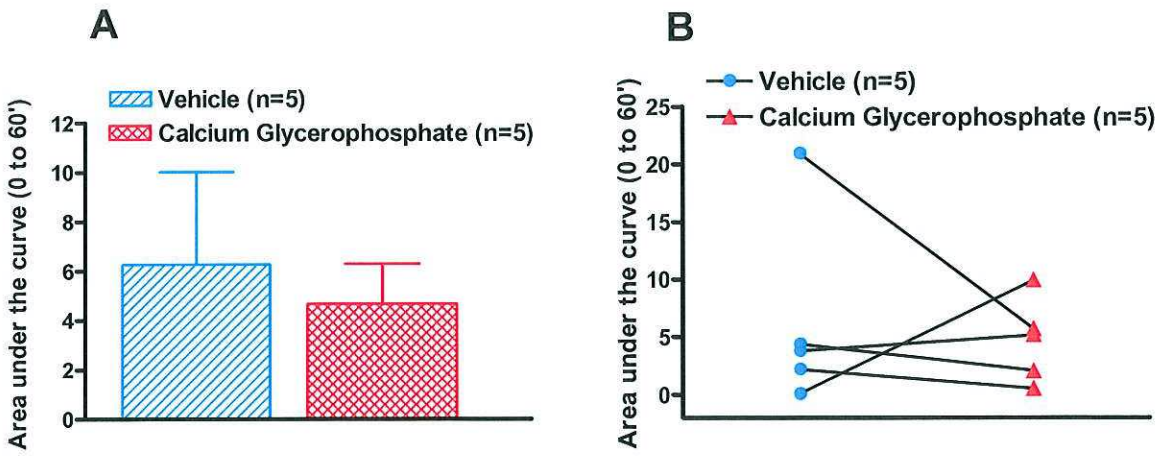

Figure 9: The area under the curve was calculated for Prostaglandin D_2 levels between 0 and 60 min (Figure 8) for vehicle and compound treatment shown as mean±sem (A, n=5) and individual data points in a scatter graph shown as change from vehicle to compound treatment (B). Prostaglandin D_2 levels decreased in three dogs and increased in two dogs and no statistical significance was reached.

9.5 Prostaglandin E_2 in nasal lavage fluid

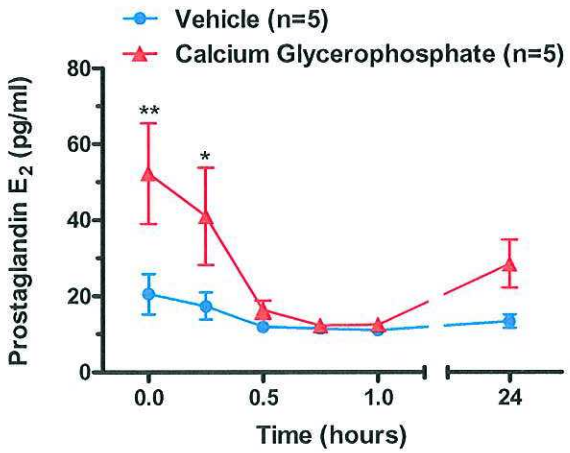

Figure 10: Prostaglandin E_2 levels in nasal lavage fluid measured before and after intranasal ragweed challenge done three days after first treatment with vehicle or compound. Data are expressed as mean±sem (n=5). T_0 indicates baseline sample collected the day before the initiation of either vehicle or calcium glycerophosphate treatment. Dogs served as their own control and vehicle treatment was compared to compound treatment. Prostaglandin E_2 levels were not statistically significant different between both treatment groups.

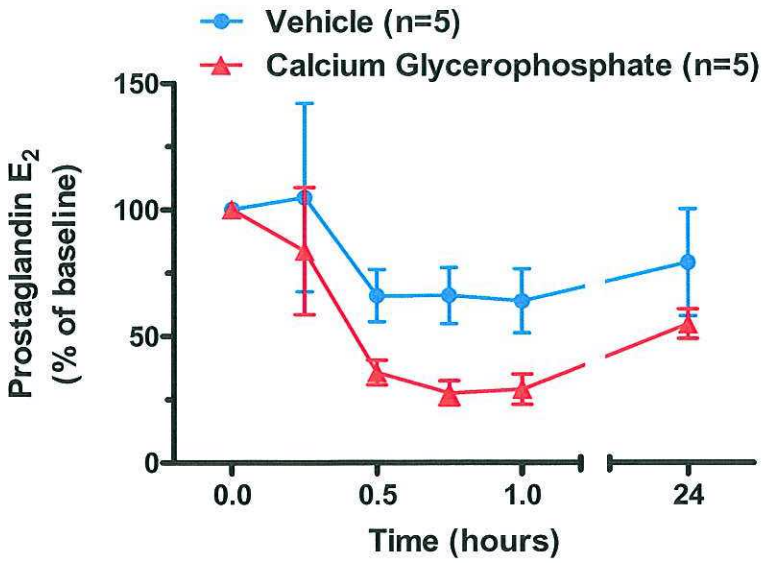

Figure 11: Due to the differences in the initial baseline levels (T_0) of PGE_2 prior to treatment the data was normalized (e.g. baseline = 100%). PGE2 levels were significantly attenuated

compared to vehicle treatment determined by Two Way Anova (p=<0.009) followed by Bonferroni posttest at individual time points (no significance at individual timepoints)

9.6 Cell Differentials in nasal lavage

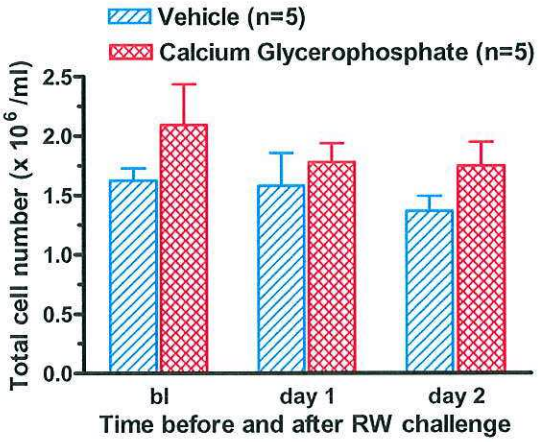

Figure 12: Total cell numbers in nasal lavage (including epithelial cells) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment.

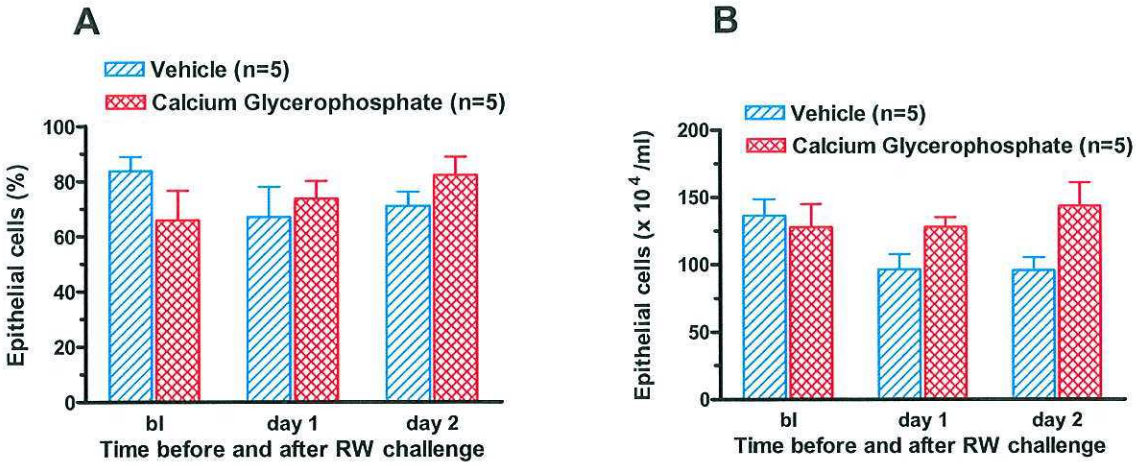

Figure 13: Epithelial cells in nasal lavage expressed as percent of cells counted (A) and as total numbers (B) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment.

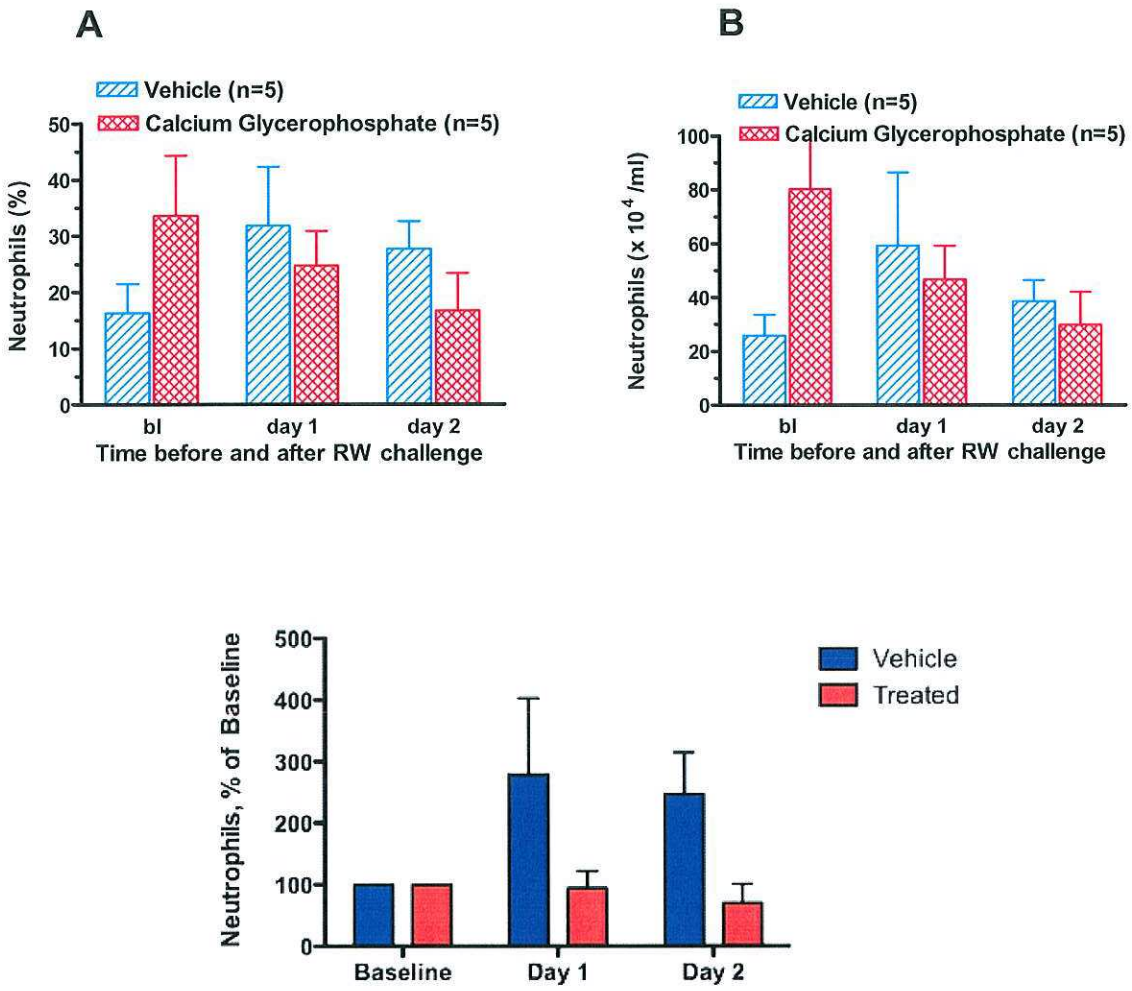

Figure 14: Neutrophils in nasal lavage expressed as percent of cells counted (A) as total numbers (B) and as percent of baseline (C) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment. When presented as percent of baseline there is a suggestive but not statistically significant effect of treatment on neutrophils.

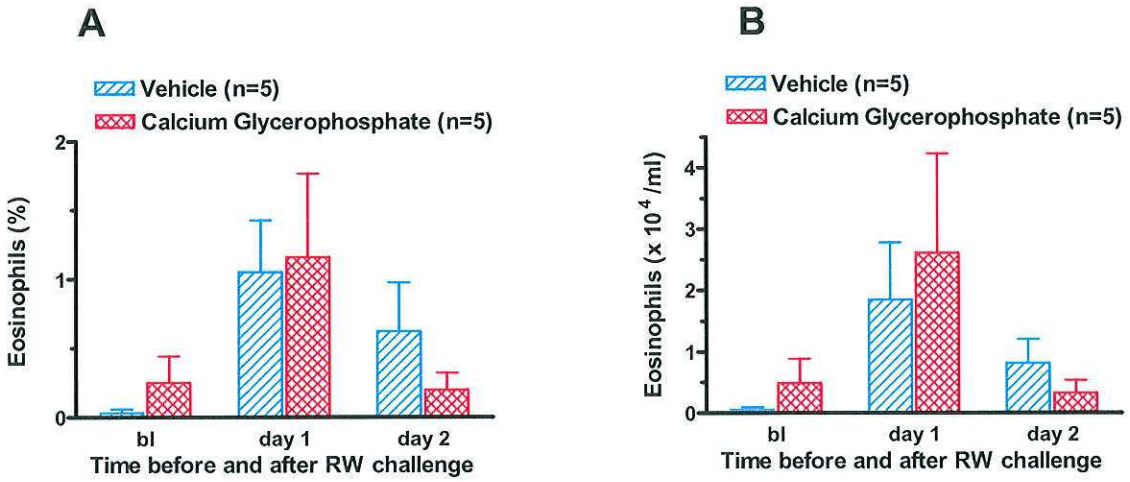

Figure 15: Eosinophils in nasal lavage expressed as percent of cells counted (A) and as total numbers (B) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment.

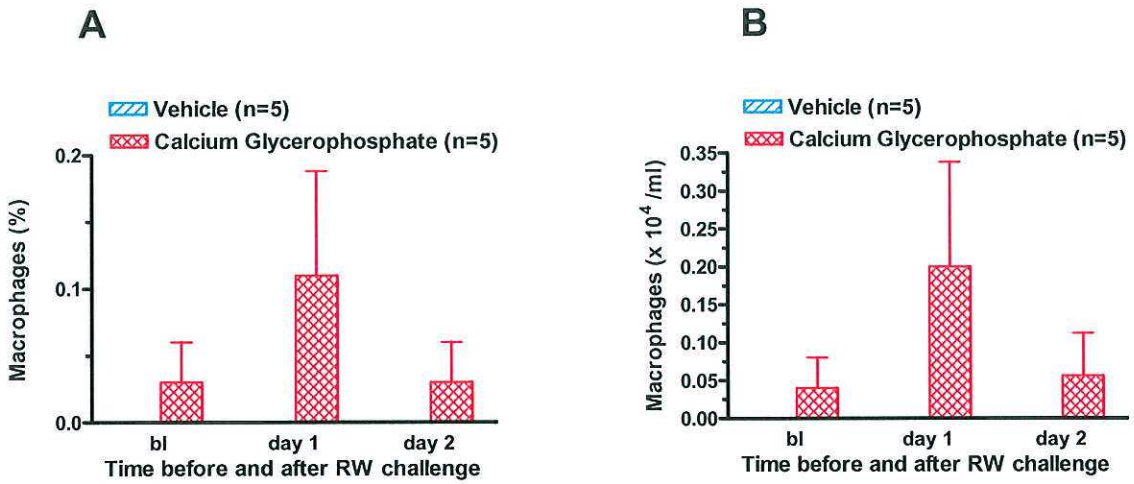

Figure 16: Macrophages in nasal lavage expressed as percent of cells counted (A) and as total numbers (B) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment.

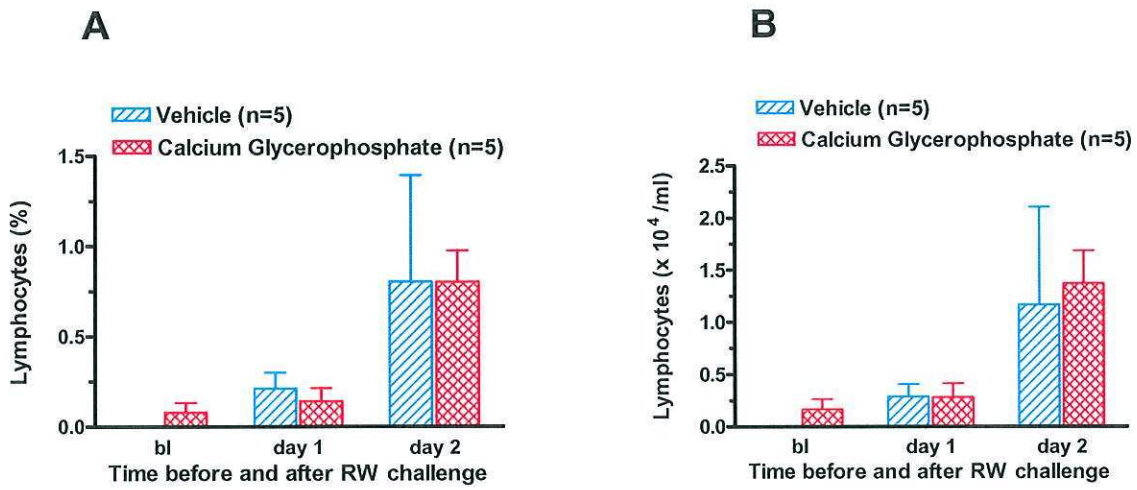

Figure 17: Lymphocytes in nasal lavage expressed as percent of cells counted (A) and as total numbers (B) collected before and after repeated oral treatment with vehicle and calcium glycerophosphate. Nasal lavages were performed prior to first treatment and on Days 1 and 2 after nasal RW challenge on 4th day of treatment.

10.0 DISCUSSION

The goal of the study was to determine whether repeated inhalation and intranasal instillation of calcium glycerophosphate attenuates ragweed induced nasal congestion and has an effect on nasal inflammation. Pretreatment with the compound at the dose achieved (1.6 mg inhaled and 30 mg intranasally instilled) significantly attenuated nasal congestion and had some minor effects on the early mediators measured in nasal lavage fluid collected for up to 60 minutes after nasal ragweed challenge. There was a general trend towards a reduction in some mediators (histamine, leukotrienes and prostaglandin D_2) but none reached statistical significance. Compound pretreatment did reduce eosinophil numbers at day 2 post ragweed challenge but this did not reach statistical significance. Treatment with calcium glycerophosphate was well tolerated by the animals and no visible adverse clinical signs were observed or any significant effects on blood chemistry parameters. Although macrophages were present in the nasal lavage following calcium glycerophosphate treatment their absolute numbers were negligible in terms of overall cells numbers.

Interestingly, PGE_2 levels were elevated in the dogs prior to the initiation of the calcium glycerophosphate treatment leg. Upon RW challenge the levels returned (T 30 – 60 min) to levels measured following the vehicle treatment leg and then were elevated at 24 h post RW challenge. PGE_2 has both inflammatory and anti-inflammatory properties. PGE_2 promotes vasodilation by activating cAMP-coupled EP2 receptors on vascular smooth muscle and increases vascular permeability indirectly by enhancing the release of histamine and other mediators from tissue leukocytes such as mast cells. As inflammation progresses, PGE_2 synthesis by macrophages is enhanced due to increased expression of COX-2 and PGE-synthase. PGE_2 inhibits leukocyte activation and promotes bronchodilation through activation of G_s-coupled EP2 and EP4 receptors (Tilley et al., 2001). The elevated levels prior to calcium glycerophosphate may be the result of the initial RW challenge during the vehicle leg, despite the 5 weeks of rest in between. When PGE_2 levels are normalized to baseline levels it suggests

that there is a greater reduction following RW challenge in conjunction with calcium glycerophosphate treatment.

We have shown previously that treatment with α-adrenergic agonist, pseudoephederine (PSE; 3 mg/kg) and histamine H1 antagonist, chlorpheniramine (10 mg/kg), in the same manner as the present study can prevent the development of RW-induced nasal congestion (Rudolph et al., 2003). The response of calcium glycerophosphate is similar to that observed previously with PSE and chlorpheneramine with respect to the attenuation of nasal congestion induced by intranasal ragweed challenge.

Similar to the present study, we have also seen variable effects of compound treatment (e.g., various new test compounds) on individual dogs with some dogs showing a negligible effect, and others showing a more dramatic effect. In general, the dogs' response to RW challenge remains consistent following repeated RW challenges, and decreases in nasal congestion are due to compound treatment and not due to variability in the response over time.

We have tested other compounds that have resulted in reduced nasal congestion in association with or without significant reductions in mediators (leukotrienes, prostaglandins, and histamine). In general, there is a high degree of variability in the peak mediator levels following RW challenge.

While we have measured statistically significant RW-induced increases in nasal lavage eosinophil numbers in some studies, generally, there is no statistically significant increase in nasal inflammatory cells. Further, to date we have not seen a profound effect of compound treatment on inflammatory cell differentials. We have not yet tested a nasally delivered steroid in this model, however, treatment with the luekotriene modifier, Montelukast, has shown similar effects (e.g. significant effect on nasal congestion with limited effect on inflammation).

Overall, the response seen following calcium glycerophosphate is similar to responses we have previously observed with an α-adrenergic agonist, pseudoephederine, a Histamine H1 antagonist, Chlorpheniramine, and Montelukast in this model.

Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes J Clin Invest. 2001 July 1; 108(1): 15–23.

Rudolph K, Bice DE, Hey JA, McLeod RL. A model of allergic nasal congestion in dogs sensitized to ragweed. *Am J Rhinol*. 2003 Jul-Aug;17(4):227-32.

11.0 APPENDIX 1 – Experimental worksheets and daily treatment times (AM and PM)

Treatment for leg 1

FY09-133

Vehicle exposure (water) + 2x 250 µl water in each nostril twice a day

day 0 to day 5 (only AM)

			baseline lavage (d -3)	RW instillation (d 3)	lavage day 4	lavage day 5
			10/2/09	10/8/09	10/9/09	10/10/09
			Friday	Thursday	Friday	Saturday
12.5hg 1656 A	Vehicle	J 04	8:15	8:54	8:40	10:35
10.25hg 1668 V	Vehicle	J 35	9:20	9:07	8:55	10:50
12.5hg 1674 S	Vehicle	J 14	8:55	10:35	10:00	11:05
10.5hg 1677 U	Vehicle	J 46	8:35	10:52	10:20	11:18
10.0hg 1678 S	Vehicle	J 46	8:50	12:16	11:20	11:35

Treatment for leg 2

FY09-133

Calcium glycerophosphate inhalation exposure + 2x 250 µl of 30 mg/ml in each nostril twice a day

day 0 to day 5 (only AM)

			baseline lavage (d -3)	RW instillation (d 3)	lavage day 4	lavage day 5	
			11/6/09	11/12/09	11/13/09	11/14/09	
			Friday	Thursday	Friday	Saturday	
10.8hg 1656 A	Compound	J 04	8:25	12:18	12:20	11:25	10.4hg
10.7hg 1668 V	Compound	J 35	8:40	8:55	9:20	8:35	10.55hg
13.0hg 1674 S	Compound	J 14	9:05	9:08	9:30	8:50	13.2hg
10:4hg 1677 U	Compound	J 46	9:20	10:49	10:38	9:35	10.4hg
9.8hg 1678 S	Compound	J 46	9:45	10:36	10:25	9:20	9.8hg

Vehicle treatment **FY09-133**

1. 15 minute inhalation exposure with PARI LC nebulizer (water)
2. intransal installation of 2x 250 µl of water in each nostril with Accuspray device

Day 0 10/05/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	8:30-8:55	ICR	2:30-3:00	ICR
1668 V	J 35	Vehicle	↓	↓	↓	↓
1674 S	J 14	Vehicle	↓	↓	↓	↓
1677 U	J 46	Vehicle	↓	↓	↓	↓
1678 S	J 46	Vehicle	↓	ICR	↓	ICR

Day 1 10/06/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	0825-0845	DRC	1423-1448	DRC
1668 V	J 35	Vehicle	↓	↓	↓	↓
1674 S	J 14	Vehicle	↓	↓	↓	↓
\	J 46	Vehicle	↓	↓	↓	↓
1678 S	J 46	Vehicle	↓	DRC	1423-1448	DRC

Day 2 10/07/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	0830-0855	DRC	① 1415-1445	DRC
1668 V	J 35	Vehicle	↓	↓	↓	↓
1674 S	J 14	Vehicle	↓	↓	↓	↓
1677 U	J 46	Vehicle	↓	↓	↓	↓
1678 S	J 46	Vehicle	↓	↓	↓	↓

Day 3 10/08/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	0825-845	DRC	1446-1510	DRC
1668 V	J 35	Vehicle	↓	↓	↓	↓
1674 S	J 14	Vehicle	↓	↓	↓	↓
1677 U	J 46	Vehicle	↓	↓	↓	↓
1678 S	J 46	Vehicle	0825-845	DRC	1446-1510	DRC

Day 4 10/09/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	0815-0845	JO	1400-1430	JO
1668 V	J 35	Vehicle	↓	↓	↓	↓
1674 S	J 14	Vehicle	↓	↓	↓	↓
1677 U	J 46	Vehicle	↓	↓	↓	↓
1678 S	J 46	Vehicle	0815-0845	JO	1400-1430	JO

Day 5 10/10/09

	location		AM time	AM initials	PM time	PM initials
1656 A	J 04	Vehicle	② 1014-1035 1400-1430	DRC		
1668 V	J 35	Vehicle	↓	↓		
1674 S	J 14	Vehicle	↓	↓		
1677 U	J 46	Vehicle	↓	↓		
1678 S	J 46	Vehicle	1014-1035	DRC		

④ Incubator error. DRC ofact 09
② wrote in wrong box. JO 09 Oct 09

Compound treatment FY09-133

1. 15 minute inhalation exposure with PARI LC nebulizer (Calcium glycerophosphate - CGP 1.5 mg)
2. intransal instillation of 2x 250 µl of 30 mg/ml CGP in each nostril with Accuspray device

			Day 0 11/09/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0908	JO	1525	JO
1668 V	J 35	CGP 1.5mg + 30mg IN	0904	JO	1521	JO
1674 S	J 14	CGP 1.5mg + 30mg IN	0906	JO	1519	JO
1677 U	J 46	CGP 1.5mg + 30mg IN	0907	JO	1517	JO
1678 S	J 46	CGP 1.5mg + 30mg IN	0905	JO	1523	JO

① wrote wrong time STM 10 Nov 09

			Day 1 11/10/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0835	JAM	~~1526~~① 1529	STM
1668 V	J 35	CGP 1.5mg + 30mg IN	0831	JAM	1523	STM
1674 S	J 14	CGP 1.5mg + 30mg IN	0833	JAM	1526	STM
16774	J 46	CGP 1.5mg + 30mg IN	0834	JAM	1525	STM
1678 S	J 46	CGP 1.5mg + 30mg IN	0835	JAM	1528	STM

			Day 2 11/11/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0845	DRC	1525	MB
1668 V	J 35	CGP 1.5mg + 30mg IN	0842	DRC	1523	DRC
1674 S	J 14	CGP 1.5mg + 30mg IN	0841	DRC	1524	MB
1677 U	J 46	CGP 1.5mg + 30mg IN	0843	DRC	1525	MB
1678 S	J 46	CGP 1.5mg + 30mg IN	0844	DRC	1526	DRC

			Day 3 11/12/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0848	JJ	1515	MB
1668 V	J 35	CGP 1.5mg + 30mg IN	0842	JJ	1512	MB
1674 S	J 14	CGP 1.5mg + 30mg IN	0844	JJ	1511	MB
1677 U	J 46	CGP 1.5mg + 30mg IN	0845	JJ	1513	MB
1678 S	J 46	CGP 1.5mg + 30mg IN	0847	JJ	1514	MB

			Day 4 11/13/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0838	JDW	1504	MB
1668 V	J 35	CGP 1.5mg + 30mg IN	0836	JDW	1504	MB
1674 S	J 14	CGP 1.5mg + 30mg IN	0837	JDW	1504	MB
1677 U	J 46	CGP 1.5mg + 30mg IN	0835	JDW	1504	MB
1678 S	J 46	CGP 1.5mg + 30mg IN	0834	JDW	1504	MB

			Day 5 11/14/09			
			AM		PM	
	location		time	initials	time	initials
1656 A	J 04	CGP 1.5mg + 30mg IN	0826	DRC		
1668 V	J 35	CGP 1.5mg + 30mg IN	0823	DRC		
1674 S	J 14	CGP 1.5mg + 30mg IN	0825	DRC		
1677 U	J 46	CGP 1.5mg + 30mg IN	0826	DRC		
1678 S	J 46	CGP 1.5mg + 30mg IN	0822	DRC		

12.0 APPENDIX 2 – Nasal Cavity volume

Nasal cavity volume (cm^3) before and after intranasal ragweed instillation

Vehicle treatment

1656 A		10/8/09 Vehicle		
	RW left nostril		ave	% change from bl
bl	8.55	7.53	8.04	**100.00**
15	4.44	4.24	4.34	**53.98**
30	4.82	4.44	4.63	**57.59**
45	4.17	4.61	4.39	**54.60**
60	4.3	4.18	4.24	**52.74**
75	4.54	5.29	4.92	**61.13**
90	4.05	4.58	4.32	**53.67**
24 h	8.56	7.06	7.81	**97.14**
48 h	8.06	7.27	7.67	**95.34**

1668 V		10/8/09 Vehicle lavage		
	RW left nostril		ave	% change from bl
bl	8.9	8.72	8.81	**100.00**
15	7.3	6.54	6.92	**78.55**
30	6.29	6.48	6.39	**72.47**
45	4.92	5.85	5.39	**61.12**
60	6.67	5.51	6.09	**69.13**
75	6.63	5.51	6.07	**68.90**
90	5.89	6.02	5.96	**67.59**
24 h	7.53	9.77	8.65	**98.18**
48 h	7.99	8.36	8.18	**92.79**

1674 S		10/8/09 Vehicle		
	RW left nostril		ave	% change from bl
bl	8.41	8.95	8.68	**100.00**
15	6.34	6.79	6.57	**75.63**
30	4.75	5.39	5.07	**58.41**
45	5.86	5.45	5.66	**65.15**
60	6.69	4.91	5.80	**66.82**
75	3.93	4.37	4.15	**47.81**
90	5.1	5.64	5.37	**61.87**
24 h	8.03	8.81	8.42	**97.00**
48 h	9.29	8.78	9.04	**104.09**

1677 U		10/8/09 Vehicle		
	RW left nostril		ave	% change from bl
bl	7.26	6.85	7.06	**100.00**
15	4.3	3.59	3.95	**55.92**
30	4.36	3.62	3.99	**56.56**
45	3.61	3.25	3.43	**48.62**
60	3.98	3.75	3.87	**54.78**
75	3.74	4.06	3.90	**55.28**
90	4.29	3.83	4.06	**57.55**
24 h	6.25	7.57	6.91	**97.94**
48 h	6.53	6.22	6.38	**90.36**

1678 S		10/8/09 Vehicle		
	RW left nostril		ave	% change from bl
bl	7.32	7.83	7.58	**100.00**
15	3.22	3.08	3.15	**41.58**
30	2.76	3.41	3.09	**40.73**
45	2.43	2.67	2.55	**33.66**
60	3.27	2.12	2.70	**35.58**
75	1.72	1.69	1.71	**22.51**
90	1.33	1.67	1.50	**19.80**
24 h	6.74	7.11	6.93	**91.42**
48 h	6.81	7.62	7.22	**95.25**

Calcium glycerophosphate treatment

1656 A		11/12/09		
	RW left nostril		ave	% change from bl
bl	8.07	7.71	7.89	**100.00**
15	6.5	5.92	6.21	**78.71**
30	6.82	5.77	6.30	**79.78**
45	6.03	6.61	6.32	**80.10**
60	6.48	6.38	6.43	**81.50**
75	6.88	5.89	6.39	**80.93**
90	6.53	6.19	6.36	**80.61**
24 h	8.71	7.22	7.97	**100.95**
48 h		7.09	7.09	**89.86**

1668 V		11/12/09		
	RW left nostril		ave	% change from bl
bl	8.72	8.54	8.63	**100.00**
15	7.43	7.24	7.34	**84.99**
30	6.92	6.89	6.91	**80.01**
45	7.32	7.24	7.28	**84.36**
60	7.76	6.74	7.25	**84.01**
75	7.46	7.32	7.39	**85.63**
90	7.25	7.58	7.42	**85.92**
24 h	8.09	8.48	8.29	**96.00**
48 h	8.09	7.09	7.59	**87.95**

1674 S		11/12/09		
	RW left nostril		ave	% change from bl
bl	8.23	8.11	8.17	**100.00**
15	6.79	6.57	6.68	**81.76**
30	8.6	8.04	8.32	**101.84**
45	8.46	7.99	8.23	**100.67**
60	7.7	7.1	7.40	**90.58**
75	8.38	7.81	8.10	**99.08**
90	8.24	7.32	7.78	**95.23**
24 h	7.49	9.17	8.33	**101.96**
48 h	7.69	7.43	7.56	**92.53**

1677 U		11/12/09		
	RW left nostril		ave	% change from bl
bl	8.24	7.41	7.83	**100.00**
15	6.72	6.13	6.43	**82.11**
30	5.74	6.13	5.94	**75.85**
45	5.93	5.76	5.85	**74.70**
60	7.15	6.43	6.79	**86.77**
75	6.76	6.74	6.75	**86.26**
90	6.73	6.92	6.83	**87.22**
24 h	7.12	7.43	7.28	**92.97**
48 h	7.24	7.63	7.44	**95.02**

1678 S		11/12/09		
	RW left nostril		ave	% change from bl
bl	7.85	7.1	7.48	**100.00**
15	6.69	6.26	6.48	**86.62**
30	5.45	5.56	5.51	**73.65**
45	7.08	6.42	6.75	**90.30**
60	6.56	6.19	6.38	**85.28**
75	7.36	7.01	7.19	**96.12**
90	6.37	6.4	6.39	**85.42**
24 h	7.69	7.56	7.63	**102.01**
48 h	7.96	8.19	8.08	**108.03**

13.0 APPENDIX 3 – Early Mediator levels in nasal lavage fluid

Mediator levels in nasal lavage fluid

		Vehicle				Calcium Glycerophosphate			
		Histamine	LTC	PGE2	PGD2	Histamine	LTC	PGE2	PGD2
		nM/ml	ng/ml	pg/ml	pg/ml	nM/ml	ng/ml	pg/ml	pg/ml
1656 A	bl	0.583	0.000	11.647	1.707	1.155	0.000	98.239	5.514
	0-15	2.325	0.077	28.883	77.797	2.529	0.000	56.779	4.197
	0-30	0.000	0.000	8.517	2.132	1.331	0.000	18.865	12.279
	0-45	0.000	0.000	9.412	2.484	1.423	0.000	12.294	2.011
	0-60	0.247	0.000	11.02	0.961	0.535	0.039	10.909	3.126
	d1	0.000	0.000	18.549	0.365	0.596	0.000	46.501	2.662
1668 V	bl	0.000	0.029	24.388	1.601	0.000	0.000	59.845	2.280
	0-15	2.666	0.034	14.926	6.735	0.076	0.006	18.858	5.842
	0-30	2.053	0.069	14.335	5.009	0.000	0.000	23.345	1.244
	0-45	14.941	0.060	11.1	0.000	0.000	0.000	13.076	0.000
	0-60	0.540	0.030	14.226	9.977	0.000	0.071	19.118	0.000
	d1	0.392	0.000	15.071	2.225	0.000	0.000	31.844	9.009
1674 S	bl	0.000	0.014	14.605	0.000	0.000	0.023	34.979	8.175
	0-15	0.261	0.039	15.609	0.000	2.249	0.140	38.763	30.255
	0-30	0.000	0.008	14.445	0.322	0.705	0.043	11.581	0.000
	0-45	0.000	0.000	11.589	0.000	0.000	0.000	9.92	3.300
	0-60	0.000	0.000	6.778	0.000	0.037	0.000	8.52	4.243
	d1	0.000	0.000	10.688	0.000	0.000	0.000	23.681	0.097
1677 U	bl	0.000	0.012	39.408	0.000	5.875	0.034	47.761	0.000
	0-15	1.388	0.000	19.919	0.000	17.063	0.019	80.775	9.694
	0-30	0.000	0.000	13.889	4.186	6.263	0.010	18.083	8.492
	0-45	53.959	0.000	13.354	9.214	5.313	0.001	18.12	0.000
	0-60	5.827	0.029	11.468	3.601	5.638	0.016	14.193	5.136
	d1	5.367	0.011	13.711	0.000	6.561	0.005	32.448	9.296
1678 S	bl	6.945	0.044	12.191	0.000	7.778	0.015	20.562	4.210
	0-15	5.851	0.000	6.944	0.002	8.471	0.000	10.126	0.000
	0-30	4.853	0.009	7.78	0.000	4.182	0.000	10.126	0.000
	0-45	4.664	0.000	11.028	4.551	4.472	0.000	7.687	0.000
	0-60	5.089	0.134	11.072	8.404	4.248	0.000	9.896	0.000
	d1	5.103	0.000	8.069	0.000	5.158	0.000	7.823	0.000

14.0 APPENDIX 4 – Area under the curve for nasal cavity volume and early mediator levels

Area under the curve for nasal congestion from 0 to 90 minutes post RW IN

Vehicle - inhalation challenge (15 min) + 2 x 250 µl water in each nostril IN
Calcium glycerophosphate - inhalation challenge (15 min) + 2 x 250 µl of 30 mg/ml in each nostril IN

Treatment twice a day starting three days prior to RW challenge
about 1.6 mg CGP inhaled + 30 mg IN

	Vehicle	Calcium glycerophosphate	% inhibition)
1656A	89.22	122.80	**55.25**
1668V	108.50	128.00	**46.99**
1674S	98.69	142.90	**86.16**
1677U	87.48	124.80	**59.69**
1678S	58.49	131.20	**79.46**
mean	88.48	129.94	65.51
sem	8.38	3.54	7.43

Area under the curve for mediators (0 - 60 minutes)

Histamine			LTC			PGE2			PGD2		
vehicle	CGP	% inhibition	vehicle	CGP	% inhibition	vehicle	CGP	% inhibition	vehicle	CGP	% inhibition
0.685	1.532	-123.65	0.019	0.005	73.68	14.54	35.63	-145.05	20.94	5.702	72.77
4.983	0.019	99.62	0.048	0.01	78.43	14.92	23.69	-58.78	4.383	2.057	53.07
0.065	0.743	-1038.85	0.014	0.049	-260.22	13.08	20.5	-56.73	0.081	9.941	-122249
14.57	8.599	40.98	0.005	0.014	-168.29	18.15	36.99	-103.80	3.8	5.189	-36.55
5.346	5.785	-8.21	0.025	0.002	92.35	9.346	10.79	-15.45	2.189	0.526	75.96

15.0 APPENDIX 5 – Total cell number and Cell differentials in nasal lavage fluid

Vehicle experiment

Total cell number x 10^6 /ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	2.05	1.76	**1.91**	1.50	1.23	**1.36**	1.91	1.63	**1.77**	1.83	1.45	**1.64**	1.34	1.51	**1.42**	1.62	0.10
day 1	10/9/09	3.33	1.98	**2.65**	1.38	1.11	**1.24**	1.45	1.51	**1.48**	1.23	1.03	**1.13**	1.58	1.15	**1.37**	1.58	0.28
day 2	10/10/09	1.65	1.87	**1.76**	1.49	1.61	**1.55**	1.35	1.14	**1.25**	1.21	0.99	**1.10**	0.80	1.50	**1.15**	1.36	0.13

Epithelial cells (%)

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	94.30	90.50	**92.40**	82.80	80.50	**81.65**	73.50	92.50	**83.00**	87.00	45.30	**66.15**	97.00	93.80	**95.40**	83.72	5.12
day 1	10/9/09	32.30	49.50	**40.90**	35.80	63.80	**49.80**	37.80	80.50	**59.15**	86.80	96.50	**91.65**	95.00	92.00	**93.50**	67.00	10.84
day 2	10/10/09	74.80	60.30	**67.55**	57.80	87.80	**72.80**	58.80	48.00	**53.40**	72.30	83.50	**77.90**	85.30	81.30	**83.30**	70.99	5.12

Neutrophils (%)

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	5.80	9.50	**7.65**	17.30	19.50	**18.40**	26.50	7.50	**17.00**	13.00	54.80	**33.90**	3.00	6.00	**4.50**	16.29	5.14
day 1	10/9/09	66.50	47.30	**56.90**	63.50	33.50	**48.50**	61.50	17.80	**39.65**	13.30	3.30	**8.30**	4.50	7.00	**5.75**	31.82	10.49
day 2	10/10/09	24.80	39.50	**32.15**	39.30	8.50	**23.90**	41.00	47.80	**44.40**	27.50	16.00	**21.75**	14.50	17.80	**16.15**	27.67	4.91

Eosinophils (%)

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.30	**0.15**	0.03	0.03
day 1	10/9/09	1.30	3.30	**2.30**	0.80	2.00	**1.40**	0.00	1.80	**0.90**	0.00	0.30	**0.15**	0.00	1.00	**0.50**	1.05	0.38
day 2	10/10/09	0.30	0.30	**0.30**	0.30	0.30	**0.30**	0.00	4.00	**2.00**	0.00	0.00	**0.00**	0.00	1.00	**0.50**	0.62	0.35

Lymphocytes (%)

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 1	10/9/09	0.00	0.00	**0.00**	0.00	0.80	**0.40**	0.80	0.00	**0.40**	0.00	0.00	**0.00**	0.50	0.00	**0.25**	0.21	0.09
day 2	10/10/09	0.00	0.00	**0.00**	2.80	3.50	**3.15**	0.30	0.30	**0.30**	0.30	0.50	**0.40**	0.30	0.00	**0.15**	0.80	0.59

Macrophages (%)

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 1	10/9/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 2	10/10/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00

Epithelial cells x 10^4 cells / ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	193.60	159.23	**176.42**	123.79	98.81	**111.30**	140.20	151.05	**145.63**	158.86	65.64	**112.25**	129.50	141.50	**135.50**	136.22	12.04
day 1	10/9/09	107.49	98.01	**102.75**	49.26	70.63	**59.94**	54.96	121.15	**88.06**	107.07	99.49	**103.28**	150.43	106.12	**128.28**	96.46	11.19
day 2	10/10/09	123.08	112.73	**117.91**	85.89	141.71	**113.80**	79.35	54.94	**67.14**	87.41	82.58	**85.00**	67.81	122.23	**95.02**	95.77	9.36

Neutrophils x 10^4 cells / ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	11.91	16.72	**14.31**	25.86	23.94	**24.90**	50.55	12.25	**31.40**	23.74	79.41	**51.57**	4.01	9.05	**6.53**	25.74	7.75
day 1	10/9/09	221.31	93.65	**157.48**	87.38	37.08	**62.23**	89.42	26.79	**58.11**	16.41	3.40	**9.90**	7.13	8.07	**7.60**	59.06	27.17
day 2	10/10/09	40.81	73.85	**57.33**	58.40	13.72	**36.06**	55.33	54.71	**55.02**	33.25	15.82	**24.54**	11.53	26.76	**19.14**	38.42	7.76

Eosinophils x 10^4 cells / ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.45	**0.23**	0.05	0.05
day 1	10/9/09	4.33	6.53	**5.43**	1.10	2.21	**1.66**	0.00	2.71	**1.35**	0.00	0.31	**0.15**	0.00	1.15	**0.58**	1.83	0.94
day 2	10/10/09	0.49	0.56	**0.53**	0.45	0.48	**0.47**	0.00	4.58	**2.29**	0.00	0.00	**0.00**	0.00	1.50	**0.75**	0.81	0.39

Lymphocytes x 10^4 cells / ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 1	10/9/09	0.00	0.00	**0.00**	0.00	0.89	**0.44**	1.16	0.00	**0.58**	0.00	0.00	**0.00**	0.79	0.00	**0.40**	0.28	0.12
day 2	10/10/09	0.00	0.00	**0.00**	4.16	5.65	**4.90**	0.40	0.34	**0.37**	0.36	0.49	**0.43**	0.24	0.00	**0.12**	1.17	0.94

Macrophages x 10^4 cells / ml

		1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	10/2/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 1	10/9/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00
day 2	10/10/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00

Calcium Glycerophosphate exposures and nasal instillation

Total cell number x 10^6 /ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	4.38	2.11	**3.25**	2.72	1.34	**2.03**	2.63	2.06	**2.35**	1.60	1.03	**1.32**	1.51	1.51	**1.51**	2.09	0.34
day 1	11/13/09	1.53	2.69	**2.11**	2.03	2.23	**2.13**	1.75	1.82	**1.78**	1.51	1.63	**1.57**	1.30	1.27	**1.29**	1.78	0.16
day 2	11/14/09	1.56	1.89	**1.73**	2.01	1.60	**1.81**	3.25	1.37	**2.31**	1.10	2.54	**1.82**	1.14	0.99	**1.07**	1.75	0.20

Epithelial cells (%)				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	55.00	62.60	**58.80**	53.00	73.00	**63.00**	49.00	56.00	**52.50**	68.00	76.00	**72.00**	70.00	69.00	**69.50**	63.16	3.54
day 1	11/13/09	75.00	70.00	**72.50**	41.00	40.00	**40.50**	68.00	53.00	**60.50**	81.00	83.00	**82.00**	85.00	82.00	**83.50**	67.80	7.96
day 2	11/14/09	85.00	78.00	**81.50**	47.00	43.00	**45.00**	85.00	73.20	**79.10**	87.00	75.70	**81.35**	93.00	84.80	**88.90**	75.17	7.72

Neutrophils (%)				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	41.00	28.80	**34.90**	28.00	22.00	**25.00**	48.00	37.00	**42.50**	31.00	21.00	**26.00**	29.00	31.00	**30.00**	31.68	3.22
day 1	11/13/09	23.00	25.00	**24.00**	51.00	51.00	**51.00**	27.00	42.00	**34.50**	19.00	13.00	**16.00**	15.00	17.00	**16.00**	28.30	6.61
day 2	11/14/09	7.00	14.00	**10.50**	48.00	49.00	**48.50**	12.00	19.20	**15.60**	9.00	20.40	**14.70**	5.00	14.10	**9.55**	19.77	7.28

Eosinophils (%)				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	0.00	1.00	**0.50**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.10	0.10
day 1	11/13/09	0.00	0.00	**0.00**	2.00	1.00	**1.50**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.30	0.30
day 2	11/14/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00

Lymphocytes (%)				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	3.00	4.80	**3.90**	18.00	4.00	**11.00**	2.00	6.00	**4.00**	1.00	3.00	**2.00**	1.00	0.00	**0.50**	4.28	1.80
day 1	11/13/09	2.00	4.00	**3.00**	3.00	7.00	**5.00**	5.00	3.00	**4.00**	0.00	2.00	**1.00**	0.00	1.00	**0.50**	2.70	0.86
day 2	11/14/09	7.00	7.00	**7.00**	5.00	5.00	**5.00**	2.00	3.80	**2.90**	4.00	3.90	**3.95**	2.00	1.10	**1.55**	4.08	0.93

Macrophages (%)				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	1.00	2.80	**1.90**	1.00	1.00	**1.00**	1.00	1.00	**1.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.78	0.36
day 1	11/13/09	0.00	1.00	**0.50**	3.00	1.00	**2.00**	0.00	2.00	**1.00**	0.00	2.00	**1.00**	0.00	0.00	**0.00**	0.90	0.33
day 2	11/14/09	1.00	1.00	**1.00**	0.00	3.00	**1.50**	1.00	3.80	**2.40**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.98	0.46

Epithelial cells x 10^4 cells / ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	240.71	132.37	**186.54**	144.13	97.75	**120.94**	128.99	115.28	**122.13**	108.80	78.32	**93.56**	105.95	104.02	**104.98**	125.63	16.12
day 1	11/13/09	114.68	188.48	**151.58**	83.07	89.26	**86.16**	119.03	96.20	**107.61**	122.67	135.50	**129.09**	110.20	104.51	**107.36**	116.36	11.12
day 2	11/14/09	132.69	147.54	**140.11**	94.61	68.69	**81.65**	276.21	100.50	**188.36**	95.79	192.62	**144.20**	105.97	84.25	**95.11**	129.89	19.06

Neutrophils x 10^4 cells / ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	179.44	60.90	**120.17**	76.15	29.46	**52.80**	126.36	76.16	**101.26**	49.60	21.64	**35.62**	43.89	46.73	**45.31**	71.03	16.70
day 1	11/13/09	35.17	67.31	**51.24**	103.33	113.81	**108.57**	47.26	76.23	**61.75**	28.78	21.22	**25.00**	19.45	21.67	**20.56**	53.42	15.82
day 2	11/14/09	10.93	26.48	**18.70**	96.62	78.28	**87.45**	38.99	26.36	**32.68**	9.91	51.91	**30.91**	5.70	14.01	**9.85**	35.92	13.54

Eosinophils x 10^4 cells / ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	0.00	2.11	**1.06**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.21	0.21
day 1	11/13/09	0.00	0.00	**0.00**	4.05	2.23	**3.14**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.63	0.63
day 2	11/14/09	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	0.00	0.00

Lymphocytes x 10^4 cells / ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	13.13	10.15	**11.64**	48.95	5.36	**27.15**	5.27	12.35	**8.81**	1.60	3.09	**2.35**	1.51	0.00	**0.76**	10.14	4.70
day 1	11/13/09	3.06	10.77	**6.91**	6.08	15.62	**10.85**	8.75	5.45	**7.10**	0.00	3.27	**1.63**	0.00	1.27	**0.64**	5.43	1.89
day 2	11/14/09	10.93	13.24	**12.08**	10.07	7.99	**9.03**	6.50	5.22	**5.86**	4.40	9.92	**7.16**	2.28	1.09	**1.69**	7.16	1.72

Macrophages x 10^4 cells / ml				1656 A			1668 V			1674 S			1677 U			1678 S		
		RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	RN	LN	ave	mean	sem
baseline	11/6/09	4.38	5.92	**5.15**	2.72	1.34	**2.03**	2.63	2.06	**2.35**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	1.90	0.95
day 1	11/13/09	0.00	2.69	**1.35**	6.08	2.23	**4.15**	0.00	3.63	**1.82**	0.00	3.27	**1.63**	0.00	0.00	**0.00**	1.79	0.67
day 2	11/14/09	1.56	1.89	**1.73**	0.00	4.79	**2.40**	3.25	5.22	**4.23**	0.00	0.00	**0.00**	0.00	0.00	**0.00**	1.67	0.80

16.0 APPENDIX 6 – Blood Chemistry and hematology data

1656 A

		Vehicle			Calcium glycerophosphate		
		2-Oct-09	8-Oct-09	10-Oct-09	6-Nov-09	12-Nov-09	14-Nov-09
	Ref. values	Baseline	Day 3	Day 5	Baseline	Day 3	Day 5
Vet Chem 20							
Glucose	*67-147*	89	77	76	79	70	83
BUN	*4.5-30.5*	13	17	14	16	15	16
Creatine		0.75	0.8	0.85	0.75	0.71	0.72
Sodium	*138-148*	142	144	142	142	140	139
Potassium	*3.5-5.0*	4.7	4.7	4.9	5.1	5	5
Chloride	*110-118*	110	110	111	111	109	111
CO2	*16-26*	24	27	26	22	23	23
Total Protein	*4.8-6.6*	6.1	5.9	5.9	5.9	5.5	5.4
Albumin	*2.3-3.9*	3.4	3.2	3.2	3	3	3
Globulin		2.7	2.7	2.7	2.8	2.5	2.5
Calcium	*9.7-12.2*	9.8	10.2	10.4	9.9	9.9	9.9
Phosphorous	*2.2-7.9*	4	4.3	4.3	4	4.7	4.6
LDH	*105-1683*	935	451	326	436	447	436
AST(SGOT)	*1.0-37.0*	49	36	35	41	37	36
ALT(SGPT)	*3.0-50.0*	45	44	61	40	39	43
GGT	*5.0-25.0*	28	12	12	13	16	14
Alk Phos	*20.0-155*	30	46	50	48	39	44
Bilirubin, Total	*0.1-0.7*	0.8	0.5	0.5	<0.1	0.1	<0.1
Bilirubin, Direct		0.6	0.4	0.4	0	0	0
Magnesium	*1.7-2.4*	2	2	2	1.9	2	2
CK	*110-118*	168	77	63	91	122	84
Triglyceride	*21-116*	37	36	32	36	33	38
CBC values							
WBC (x103)	*5.5-17*	8.9	9.7	9.1	10.1	10.1	9.4
RBC (x106)	*5.5-8.5*	8.68	8.52	7.83	8.43	8.27	8.03
Hgb (g/dL)	*12.0-18.0*	20.2	19.3	18.7	19.6	19.6	18.9
Hct (%)	*37-55*	60	57	53	56	56	54
MCV (fL)	*60-73*	68	69	68	67	67	68
MCHC (g/dL)	*31-38*	33.9	33.7	35.3	34.9	35.1	34.8
Platelets (x103)	*175-500*	218	249	263	260	248	249
Differentials							
Neutrophils (%)	*60-77*	73	52	59	64	66	63
Lymphocytes (%)	*12.0-30.0*	17	30	34	26	22	31
Variant Lymph (%)							
Monocytes (%)	*3.0-10.0*	4	6	4	7	12	5
Eosinophils (%)	*2.0-10.0*	6	12	3	3	0	1
Basophils (%)	*0-1*	0	0	0	0	0	0
Abs Neutrophils (x103)	*3.0-12.0*	6.5	5	5.3	6.5	6.7	5.9
Abs Lymphocytes (x(103)	*1.0-4.9*	1.5	2.9	3.1	2.6	2.2	2.9
Abs. Monocyte (x103)	*0.1-1.4*	0.4	0.6	0.4	0.7	1.2	0.5
Abs. Eosinophil (x103)	*0.1-1.5*	0.5	1.2	0.3	0.3	0	0.1
Abs. Basophil (x103)	*< 0.1*	0	0	0	0	0	0

1668 V

		Vehicle			Calcium glycerophosphate		
		2-Oct-09	8-Oct-09	10-Oct-09	6-Nov-09	12-Nov-09	14-Nov-09
	Ref. values	Baseline	Day 3	Day 5	Baseline	Day 3	Day 5
Vet Chem 20							
Glucose	*67-147*	90	68	72	77	82	88
BUN	*4.5-30.5*	12	11	19	16	14	15
Creatine		0.76	0.76	0.75	0.73	0.71	0.74
Sodium	*138-148*	140	143	143	141	141	140
Potassium	*3.5-5.0*	4.6	4.8	4.9	4.7	4.8	4.4
Chloride	*110-118*	112	112	111	112	110	112
CO2	*16-26*	22	25	26	21	23	24
Total Protein	*4.8-6.6*	6.1	6.7	6.1	5.9	6.1	5.7
Albumin	*2.3-3.9*	3.1	3.3	3	2.8	3.1	2.8
Globulin		3	3.4	3.1	3	3	2.8
Calcium	*9.7-12.2*	10.6	10.1	10.6	10.4	10.3	10.4
Phosphorous	*2.2-7.9*	3.8	3.6	5.3	5.1	4	3.8
LDH	*105-1683*	336	743	362	352	416	241
AST(SGOT)	*1.0-37.0*	27	44	30	34	37	26
ALT(SGPT)	*3.0-50.0*	30	37	33	29	40	25
GGT	*5.0-25.0*	<10	25	<10	10	<10	10
Alk Phos	*20.0-155*	137	187	119	97	106	122
Bilirubin, Total	*0.1-0.7*	0.1	1.5	0.5	<0.1	0.1	<0.1
Bilirubin, Direct		0.1	1.2	0.5	0	0	0
Magnesium	*1.7-2.4*	1.8	1.9	1.9	1.9	1.9	1.8
CK	*110-118*	60	183	79	190	218	72
Triglyceride	*21-116*	29	35	59	45	36	34
CBC values							
WBC (x103)	*5.5-17*	11.6	8	10.7	9.9	9.3	9.8
RBC (x106)	*5.5-8.5*	7.43	7.53	7.82	7.18	7.6	7.42
Hgb (g/dL)	*12.0-18.0*	17.7	18.3	19	17.2	18	17.6
Hct (%)	*37-55*	52	53	55	50	52	51
MCV (fL)	*60-73*	70	70	70	70	68	69
MCHC (g/dL)	*31-38*	34.1	34.4	34.7	34.2	34.9	34.3
Platelets (x103)	*175-500*	290	337	381	293	236	311
Differentials							
Neutrophils (%)	*60-77*	81	66	60	66	78	55
Lymphocytes (%)	*12.0-30.0*	13	26	33	26	19	38
Variant Lymph (%)							
Monocytes (%)	*3.0-10.0*	5	8	6	6	3	5
Eosinophils (%)	*2.0-10.0*	1	0	1	2	0	2
Basophils (%)	*0-1*	0	0	0	0	0	0
Abs Neutrophils (x103)	*3.0-12.0*	9.4	5.3	6.5	6.5	7.2	5.4
Abs Lymphocytes (x(103)	*1.0-4.9*	1.5	2.1	3.5	2.6	1.8	3.7
Abs. Monocyte (x103)	*0.1-1.4*	0.6	0.6	0.6	0.6	0.3	0.5
Abs. Eosinophil (x103)	*0.1-1.5*	0.1	0	0.1	0.2	0	0.2
Abs. Basophil (x103)	*< 0.1*	0	0	0	0	0	0

1674 S

		Vehicle			Calcium glycerophosphate		
		2-Oct-09	8-Oct-09	10-Oct-09	6-Nov-09	12-Nov-09	14-Nov-09
	Ref. values	Baseline	Day 3	Day 5	Baseline	Day 3	Day 5
Vet Chem 20							
Glucose	*67-147*	82	84	77	64	74	81
BUN	*4.5-30.5*	15	14	13	15	12	13
Creatine		0.73	0.76	0.74	0.85	0.68	0.67
Sodium	*138-148*	144	141	142	142	139	141
Potassium	*3.5-5.0*	4.7	4.9	4.8	4.8	4.7	4.7
Chloride	*110-118*	112	114	115	110	111	115
CO2	*16-26*	23	24	23	25	24	22
Total Protein	*4.8-6.6*	5.9	5.3	5.8	5.4	5.3	5.6
Albumin	*2.3-3.9*	3.2	2.9	3.1	2.9	2.7	2.7
Globulin		2.7	2.4	2.8	2.5	2.6	2.8
Calcium	*9.7-12.2*	10.7	10.4	10.2	10.4	10.2	10.3
Phosphorous	*2.2-7.9*	4.4	4.1	4.3	3.6	4.5	4.3
LDH	*105-1683*	578	484	328	636	455	422
AST(SGOT)	*1.0-37.0*	37	37	39	39	30	29
ALT(SGPT)	*3.0-50.0*	46	53	59	41	32	45
GGT	*5.0-25.0*	11	<10	<10	11	<10	<10
Alk Phos	*20.0-155*	108	94	86	72	79	72
Bilirubin, Total	*0.1-0.7*	0.1	<0.1	0.4	<0.1	0.1	<0.1
Bilirubin, Direct		0.1	0	0.4	0	0	0
Magnesium	*1.7-2.4*	2.1	2.1	2	2.1	2.1	2
CK	*110-118*	88	112	149	128	146	82
Triglyceride	*21-116*	31	37	38	25	46	40
CBC values							
WBC (x103)	*5.5-17*	9.7	9.4	9.2	11.2	14.7	19.6
RBC (x106)	*5.5-8.5*	7.76	7.65	7.6	7.38	7.35	7.28
Hgb (g/dL)	*12.0-18.0*	17.8	17.9	17.7	17.8	17.2	17.1
Hct (%)	*37-55*	53	53	52	51	51	51
MCV (fL)	*60-73*	68	69	68	69	69	71
MCHC (g/dL)	*31-38*	33.7	33.9	34	34.8	34	33.2
Platelets (x103)	*175-500*	319	345	336	324	293	378
Differentials							
Neutrophils (%)	*60-77*	63	57	47	67	49	57
Lymphocytes (%)	*12.0-30.0*	31	33	45	26	22	34
Variant Lymph (%)							2
Monocytes (%)	*3.0-10.0*	4	8	4	4	8	5
Eosinophils (%)	*2.0-10.0*	2	2	4	3	1	2
Basophils (%)	*0-1*	0	0	0	0	0	0
Abs Neutrophils (x103)	*3.0-12.0*	6.1	5.3	4.3	7.6	7.2	11.1
Abs Lymphocytes (x(103)	*1.0-4.9*	3	3.1	4.1	2.9	6.2	7.1
Abs. Monocyte (x103)	*0.1-1.4*	0.4	0.8	0.4	0.4	1.2	1
Abs. Eosinophil (x103)	*0.1-1.5*	0.2	0.2	0.4	0.3	0.1	0.4
Abs. Basophil (x103)	*< 0.1*	0	0	0	0	0	0
NRBC (/100WBC)						2	

1677 U

		Vehicle			Calcium glycerophosphate		
		2-Oct-09	8-Oct-09	10-Oct-09	6-Nov-09	12-Nov-09	14-Nov-09
	Ref. values	Baseline	Day 3	Day 5	Baseline	Day 3	Day 5
Vet Chem 20							
Glucose	*67-147*	92	88	89	75	84	86
BUN	*4.5-30.5*	15	12	13	15	13	13
Creatine		0.69	0.66	0.66	0.59	0.53	0.59
Sodium	*138-148*	144	143	144	144	139	141
Potassium	*3.5-5.0*	4.5	4.5	4.5	4.7	4.6	4.4
Chloride	*110-118*	114	115	114	112	111	112
CO2	*16-26*	22	23	26	25	22	23
Total Protein	*4.8-6.6*	5.4	5.5	5.6	5.1	6.1	5.6
Albumin	*2.3-3.5*	2.9	3	2.9	2.6	3.2	2.9
Globulin		2.5	2.5	2.7	2.5	2.9	2.7
Calcium	*9.7-12.2*	9.9	10.4	10.2	9.9	9.5	10
Phosphorous	*2.2-7.5*	3.7	3.4	4.2	4.2	3.8	3.8
LDH	*105-1683*	359	476	261	413	1153	568
AST(SGOT)	*1.0-37.0*	43	46	44	45	63	47
ALT(SGPT)	*3.0-50.0*	65	223	149	61	52	60
GGT	*5.0-25.0*	11	11	<10	12	16	10
Alk Phos	*20.0-155*	63	76	75	75	81	86
Bilirubin, Total	*0.1-0.7*	<0.1	<0.1	0.4	<0.1	1.2	0.4
Bilirubin, Direct		0	0	0.4	0	1.1	0.4
Magnesium	*1.7-2.4*	1.9	2.1	2.1	2	1.9	1.9
CK	*110-118*	147	135	135	232	266	191
Triglyceride	*21-116*	36	37	52	30	51	44
CBC values							
WBC (x103)	*5.5-17*	7.9	7.4	9.6	9.1	9.3	10.7
RBC (x106)	*5.5-8.5*	7.21	7.31	7.27	5.86	6.55	7.1
Hgb (g/dL)	*12.0-18.0*	17.2	17.7	17.5	14.3	15.8	17.3
Hct (%)	*37-55*	50	51	50	40	45	50
MCV (fL)	*60-73*	69	70	68	69	69	71
MCHC (g/dL)	*31-38*	34.5	34.7	35.2	35.3	35	34.5
Platelets (x103)	*175-500*	335	367	357	299	398	398
Differentials							
Neutrophils (%)	*60-77*	47	65	62	50	53	59
Lymphocytes (%)	*12.0-30.0*	48	29	34	41	39	39
Variant Lymph (%)							
Monocytes (%)	*3.0-10.0*	3	6	3	6	8	2
Eosinophils (%)	*2.0-10.0*	2	0	1	3	0	0
Basophils (%)	*0-1*	0	0	0	0	0	0
Abs Neutrophils (x103)	*3.0-12.0*	3.7	4.9	5.9	4.6	5	6.3
Abs Lymphocytes (x(103)	*1.0-4.9*	3.8	2.1	3.3	3.7	3.6	4.2
Abs. Monocyte (x103)	*0.1-1.4*	0.2	0.4	0.3	0.5	0.7	0.2
Abs. Eosinophil (x103)	*0.1-1.5*	0.2	0	0.1	0.3	0	0
Abs. Basophil (x103)	*< 0.1*	0	0	0	0	0	0
NRBC (/100WBC)						1	1

1678 S

		Vehicle			Calcium glycerophosphate		
		2-Oct-09	8-Oct-09	10-Oct-09	6-Nov-09	12-Nov-09	14-Nov-09
	Ref. values	Baseline	Day 3	Day 5	Baseline	Day 3	Day 5
Vet Chem 20							
Glucose	*67-147*	93	77	77	78	84	75
BUN	*4.5-30.5*	13	13	13	16	13	13
Creatine		0.84	0.88	0.82	0.75	0.72	0.78
Sodium	*138-148*	143	143	142	141	139	139
Potassium	*3.5-5.0*	4.4	4.7	4.7	4.3	4.1	4.5
Chloride	*110-118*	114	111	112	109	111	109
CO2	*16-26*	22	25	25	27	23	25
Total Protein	*4.8-6.6*	5.8	5.9	6	5.9	5.6	6.2
Albumin	*2.3-3.9*	3.2	3.1	3.1	2.9	3	3
Globulin		2.6	2.8	2.9	2.9	2.6	3.2
Calcium	*9.7-12.2*	10.4	10.5	10.2	10.4	10.1	10.1
Phosphorous	*2.2-7.9*	3.4	3.5	3.9	4.6	3.8	3.7
LDH	*105-1683*	213	388	390	391	323	441
AST(SGOT)	*1.0-37.0*	30	114	53	34	29	32
ALT(SGPT)	*3.0-50.0*	49	81	73	37	44	45
GGT	*5.0-25.0*	<10	<10	11	11	<10	12
Alk Phos	*20.0-155*	69	85	82	104	100	98
Bilirubin, Total	*0.1-0.7*	0.1	0.4	0.5	<0.1	0.1	<0.1
Bilirubin, Direct		0.1	0.4	0.5	0	0	0
Magnesium	*1.7-2.4*	1.9	1.9	1.9	2	1.8	1.9
CK	*110-118*	67	1909	339	70	76	91
Triglyceride	*21-116*	28	26	36	24	35	34
CBC values							
WBC (x103)	*5.5-17*	4.7	5.1	4.9	8	8.8	8.1
RBC (x106)	*5.5-8.5*	6.99	7.49	7.75	7.26	7.49	7.25
Hgb (g/dL)	*12.0-18.0*	16.5	17.9	18.1	17.2	17.5	17
Hct (%)	*37-55*	49	53	55	50	50	49
MCV (fL)	*60-73*	69	70	70	68	67	68
MCHC (g/dL)	*31-38*	34	34	33.3	34.7	34.8	34.3
Platelets (x103)	*175-500*	250	200	257	235	268	211
Differentials							
Neutrophils (%)	*60-77*	69	72	49	70	83	60
Lymphocytes (%)	*12.0-30.0*	24	21	46	21	14	28
Variant Lymph (%)							
Monocytes (%)	*3.0-10.0*	2	6	4	8	3	8
Eosinophils (%)	*2.0-10.0*	5	1	1	1	0	4
Basophils (%)	*0-1*	0	0	0	0	0	0
Abs Neutrophils (x103)	*3.0-12.0*	3.3	3.6	2.3	5.6	7.3	4.9
Abs Lymphocytes (x(103)	*1.0-4.9*	1.1	1.1	2.3	1.7	1.2	2.3
Abs. Monocyte (x103)	*0.1-1.4*	0.1	0.3	0.2	0.6	0.3	0.6
Abs. Eosinophil (x103)	*0.1-1.5*	0.2	0.1	0.1	0.1	0	0.3
Abs. Basophil (x103)	*< 0.1*	0	0	0	0	0	0
NRBC (/100WBC)							2

17.0 APPENDIX 7 – Inhalation Exposure Report

INHALATION EXPOSURE REPORT

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Prepared for
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Date

Executive Summary

Inhalation exposures to calcium glycerophospate (Lot: CGP0060109, 20 mg/mL solution) and vehicle (ultra pure water) were conducted in a canine face mask exposure system. Aerosols were generated with two Pari LC Plus nebulizer operated in tandem at an inlet pressure of 20 psi. Fifteen minute exposures were conducted twice a day (am and pm) for vehicle (05 Oct 2009 to 10 Oct 2009) and for test article (09 Nov 2009 to 14 Nov 2009). The aerosol concentrations were monitored directly from the breathing zone of the nose-only exposure port by collection onto a filter. Filters were analyzed gravimetrically by differential weight analysis to determine the total aerosol concentration. The average total aerosol concentration for the test article exposures was 0.17 mg/L. The average total aerosol concentration for the placebo exposures was 0.00 mg/L. The particle size, determined by a mercer seven stage impactor, was measured to be 1.36 μm MMAD with a GSD of 1.82 for the test article.

Description of Exposure System

A schematic of the aerosol exposure system is shown in Figure 1, both test article and vehicle aerosol systems were set up the same. Aerosols (test article and / or vehicle) were generated with two Pari LC Plus compressed air jet nebulizers with an inlet pressure of 20 psi. The test article was formulated at 20 mg/mL (solution) in ultra pure water and the vehicle was ultra pure water. Aerosols were directed through approximately 24 in. of a 1.58-cm (diameter) delivery line. Aerosols transited into a distribution plenum that allowed for the aerosols to be individually directed to each canine. Exhaust flow was maintained at ~ 5 L/min with critical orfice on each distribution line.

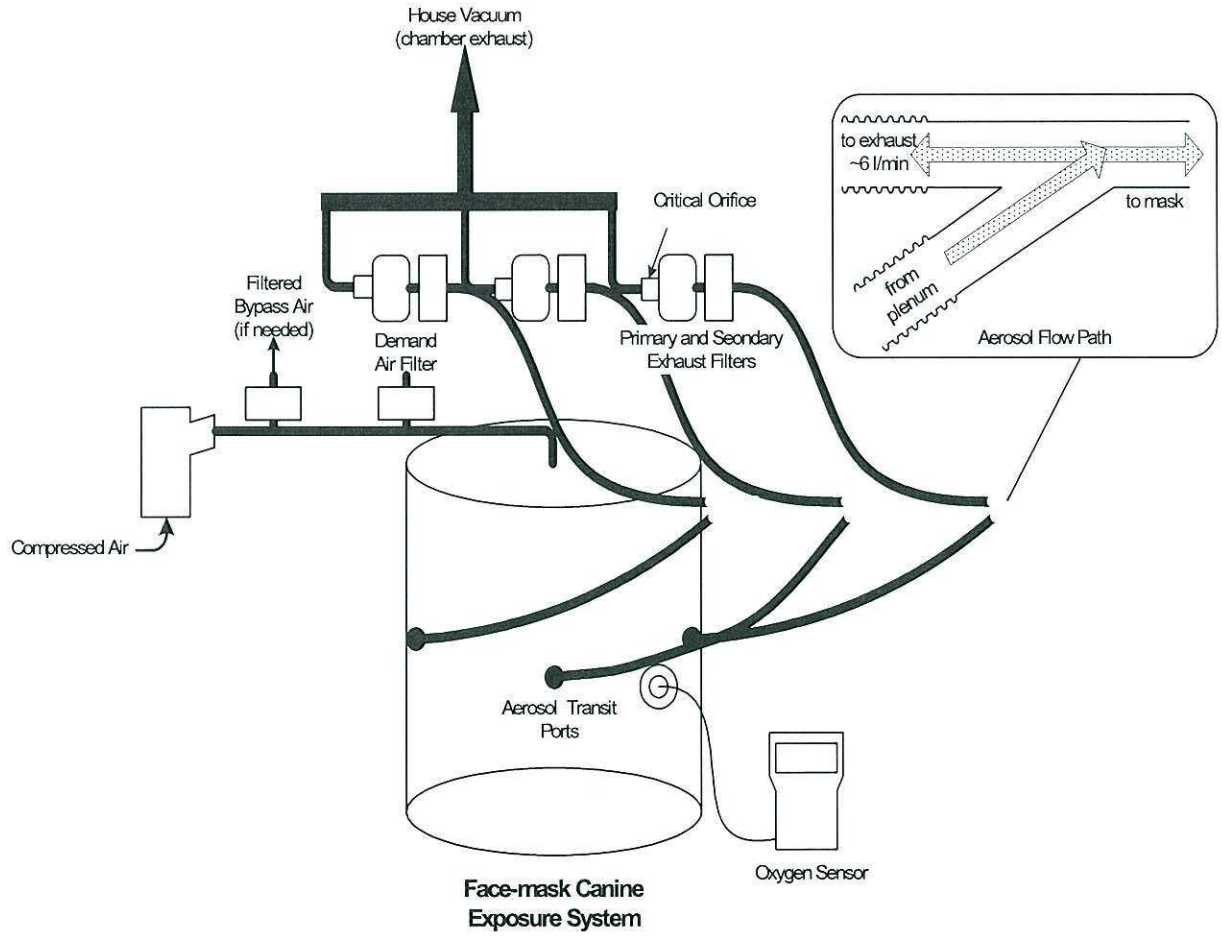

Figure 1: Schematic of the exposure system for FY09-133.

Distribution chamber oxygen content was monitored throughout each exposure. Temperature readings were obtained from a monitor placed next to the exposure chamber.

All flows and system pressures (e.g., pressure in sample, exhaust, and pressure lines) were monitored by both rotameter (flow) and magnahelics (pressure). The rotameters and magnahelics were enclosed in a control panel. Flows were point-calibrated (calibrated at a specific flow rate) with a calibrated flow meter.

Exposure Monitoring for Calcium Glycerophospate Concentration and Particle Size

Exposure monitoring was conducted by collecting material onto Pallflex 47-mm filters (Pallflex) at a flow rate of 2.0 L/min in order to collect sufficient material for chemical analysis. Material was sampled from a breathing line that was set up identical to the lines used for each canine. After collection, the filters were analyzed gravimetrically to determine the total aerosol concentration.

The particle size, measured by Mercer-style seven-stage cascade impactor (IN-TOX Products, Inc., Albuquerque, NM), was collected at the breathing zone of the exposure system at a flow rate of 2.0 L/min. Impactor data were analyzed to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD).

Deposited Dose Calculation

In order to calculate the deposited dose to each animal Eqn 1 was used with an assumed deposition fraction of 20%. In this manner the amount of test article that deposited in the lungs was calculated using the actual aerosol concentration measured during the exposures.

$$DD\ (ug) = \frac{AC\ (mg/L) \times RMV\ (L/min) \times T\ (min)}{DF} \qquad \text{Eqn. 1}$$

Where:

Inhaled Dose = (DD)

Mouse minute volume (RMV) = Estimated (0.025 L/min, Bide, R.W., Armour, S. J., and Yee, E., Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans. *J. Appl. Toxicol.* 20(4):273-290, 2000)

Aerosol exposure concentration (AC) = Measured from Exposure Chamber

Deposition Fraction (DF) = assumed deposition fraction of 20%

Results

Exposure Atmosphere Monitoring for Inhalation Exposures

All exposure filters were analyzed gravimetrically to determine both the total aerosol concentration. The results for the vehicle exposures are shown in Table 1 and the test article results are shown in Table 2. The average aerosol concentration for the test article of 0.17 mg/L results in a calculated deposited dose to the lungs of 1.64 mg for each animal.

Table 1. Average daily total aerosol concentration for each day of the vehicle exposures

Date	Exposure Group	Total Aerosol Conc. (mg/L)
05 Oct 2009	AM	0.00
05 Oct 2009	PM	0.00
06 Oct 2009	AM	0.00
06 Oct 2009	PM	0.00
07 Oct 2009	AM	0.00
07 Oct 2009	PM	0.00
08 Oct 2009	AM	0.00
08 Oct 2009	PM	0.00
09 Oct 2009	AM	0.00
09 Oct 2009	PM	0.00
10 Oct 2009	AM	0.00
	Avg	0.00

Table 2. Average daily total aerosol concentration for each day of the test article exposures

Date	Exposure Group	Total Aerosol Conc. (mg/L)
09 Nov 2009	AM	0.17
09 Nov 2009	PM	0.15
10 Nov 2009	AM	0.16
10 Nov 2009	PM	0.16
11 Nov 2009	AM	0.16
11 Nov 2009	PM	0.13
12 Nov 2009	AM	0.17
12 Nov 2009	PM	0.15
13 Nov 2009	AM	0.19
13 Nov 2009	PM	0.18
14 Nov 2009	AM	0.21
	Avg	0.17

Particle Size Analysis during Study FY09-133

Particle size analysis of calcium glycerophospate was conducted once for each during the study. Impactor data were analyzed to provide MMAD and GSD. Data were processed in accordance with LRRI standard operating procedures for reduction of impactor data (data summarized in Table 2). Histogram for the impactor analysis is shown in Figure 2

Table 2. Particle size and GSD for calcium glycerophospate during Study FY09-133

Date	MMAD (μm)	GSD
14 Nov 2009	1.36	1.82

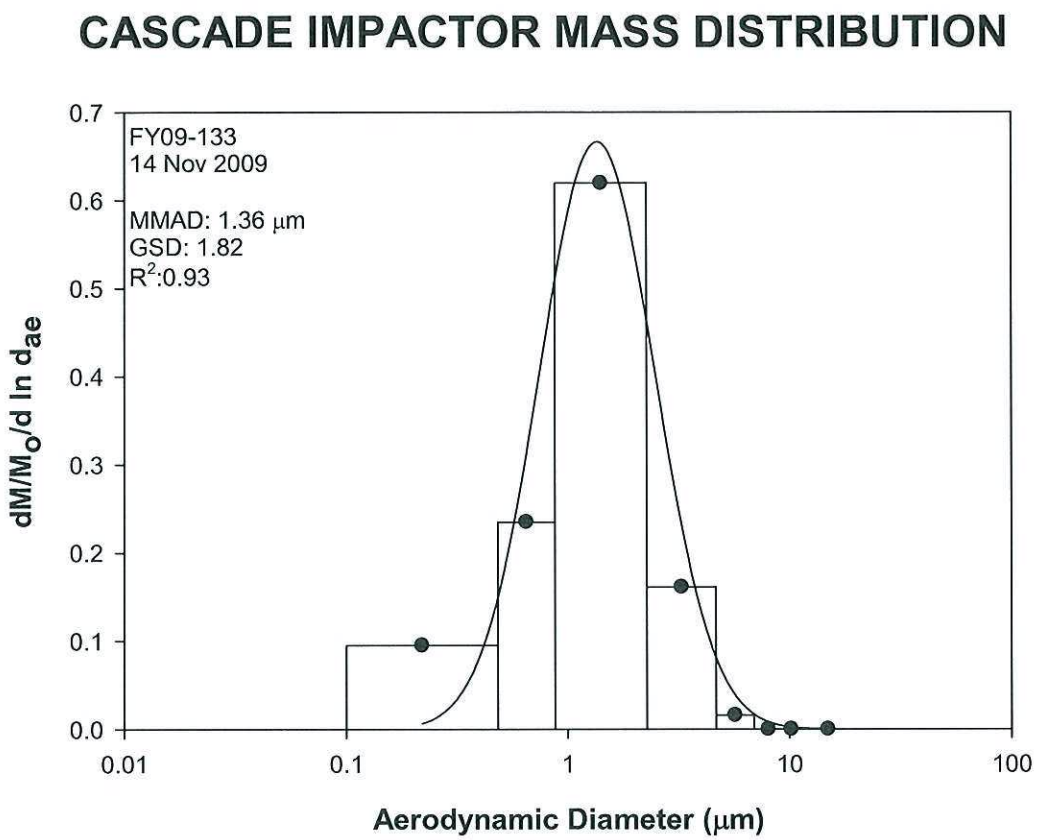

Figure 2. Histogram of test article impactor from FY09-133.