USE OF OSELTAMIVIR IN THE TREATMENT OF CANINE PARVOVIRAL ENTERITIS

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USE OF OSELTAMIVIR IN THE TREATMENT OF CANINE PARVOVIRAL ENTERITIS

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THESIS ABSTRACT

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Despite the availability of an effective vaccine, canine parvovirus (CPV) enteritis remains a significant cause of disease in veterinary medicine. Appropriate treatment of the disease can result in a favorable survival rate; however, lack of treatment is almost universally fatal. Unfortunately, the treatment tends to be costly to the client, often times deterring medical care. There exists a need for an effective treatment option that will ameliorate disease morbidity in addition to hospitalization time, thereby decreasing client cost. Oseltamivir, a neuraminidase inhibitor antiviral drug, has resounding anecdotal support for achieving this goal. However, scientific validation at this point is lacking. This study, using a prospective, blinded, randomized, placebo controlled clinical trial, aimed to investigate the effects of oseltamivir as an adjunct to standard treatment of CPV enteritis. Results show the only significant difference between groups to be in weight change during hospitalization in the oseltamivir group versus placebo.

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INTRODUCTION

Canine parvovirus (CPV) is a single-stranded DNA virus that was first discovered in 1978.[1] It is a hardy, highly contagious virus that remains a cause of significant disease process in young dogs. It is estimated that over one million dogs are affected each year in the United States[2] despite the availability of an effective vaccine.

CPV infects and replicates in rapidly dividing cells, most notably the lymphoid organs, latter myeloid progenitor cells in the bone marrow, and intestinal epithelial cells. Replication results in cell destruction, causing a clinical disease characterized by severe vomiting, hemorrhagic diarrhea, dehydration and neutropenia. This disease is almost universally fatal without treatment, with reported survival rates of only 9% in an experimental model.[3] Treatment increases this figure significantly, with reported survival rates ranging from 64-95%.[2, 4-10] Care given in a university setting may be associated with increased survival rates of 96-100%[2, 8], versus 67-75%[2, 7] at local day clinics. It is postulated that this increase may be due to the 24 hour care available at these universities, as well as the likelihood for more intensive treatments such as plasma or colloid therapy.[2]

Therapy for CPV enteritis remains mainly supportive and aimed at controlling the clinical signs of disease. Multiple more directed therapies have been studied, such as human recombinant granulocyte colony stimulating factor[8, 9, 11], equine antiendotoxin[7, 12], recombinant bactericidal/permeability-increasing protein[2], and interferon omega[13, 14] with variable or disappointing results. Early enteral nutrition has been the only modality to

date to show promise for a shortened recovery time and decreased disease morbidity associated with CPV enteritis.[10] However, the need still remains for a therapeutic agent that will help ameliorate the morbidity and mortality of this disease, and in doing so also decrease the cost of treatment. While the survival rate with aggressive treatment is very good, financial constraints often result in suboptimal treatment with lower survival, or even euthanasia. This fact demonstrates the clear need for an agent to help make the appropriate treatment for CPV enteritis a more financially feasible option for many owners or shelters.

Oseltamivir[a] (Tamiflu) is a neuraminidase inhibitor originally designed to treat human influenza virus.[15] It has recently also shown efficacy in the treatment of avian influenza. Oseltamivir inhibits the viral neuraminidase enzyme, and thus prevents the cleavage of sialic acid residues. This cleavage is necessary for liberating newly formed virons from the host cell, as well as for preventing the aggregation of viral particles. Both the viron liberation and decreased aggregation are mechanisms necessary for the spread and dissemination of the virus throughout the host in order to further infection. The neuraminidase enzyme is also needed for the virus to cleave the sialic acid residues in mucin to allow for penetration of this protective layer and infection of respiratory epithelial tissue.

Anecdotal reports of oseltamivir use in veterinary medicine claim that it is associated with a less severe form of disease and a quicker recovery when administered to dogs with CPV enteritis. However, unlike the influenza virus, CPV does not rely on neuraminidase for effective replication. Therefore, any beneficial effects that may be present would not be due to a direct antiviral action. Human studies have shown a

significant decrease in the development of bacterial infections secondary to influenza when oseltamivir is used.[15-21] This effect is believed to be due to a decrease in bacterial permeation through the mucin layer of the respiratory epithelial cells. An extension of this is that oseltamivir could also have a similar inhibitory effect on bacterial permeation through the mucin layer of the gut epithelial cells. This would decrease bacterial translocation, resulting in a potentially lower incidence of endotoxemia, sepsis, systemic inflammatory response and eventual organ failure that is thought to be the main mechanism behind the mortality of CPV enteritis. Thus, oseltamivir could have an effect on the disease process of CPV enteritis in a non-viral dependent manner. In one report, 90% of dogs that died from CPV enteritis had *Escherichia coli* cultured from their liver and lungs.[22] ARDS-like changes in the lungs of these dogs on histopathologic exam was also present, demonstrating the possibility for organ failure secondary to SIRS development.[22]

This study was designed to investigate the influence of oseltamivir when added to standard treatment on the disease process of CPV enteritis, as well as to monitor for significant adverse side effects associated with its use. Our hypothesis was that oseltamivir would help to ameliorate the disease morbidity and mortality associated with naturally occurring CPV enteritis, thereby decreasing hospitalization time, the need for colloid and other adjunctive therapies, and thus the cost associated with treatment.

LITERATURE REVIEW

Canine parvovirus (CPV) is a single-stranded, non-enveloped DNA virus that infects and destroys rapidly dividing cells of the host. It is a highly contagious and hardy

virus, resistant to environmental conditions and many cleaning agents. The virus is transmitted via the fecal-oral route. It primarily affects young dogs six weeks to six months old, with the majority of older dogs showing immunity from either natural infection or vaccination. CPV has a propensity to infect rapidly dividing cells. These cells in dogs over eight weeks old include those of lymphoid organs, the latter myeloid progenitor cells in the bone marrow, and intestinal epithelial cells. Rarely, the lungs, liver and/or kidney can be infected. Dogs younger than eight weeks or pups in utero can also have the virus infect myocardial cells, resulting in a myocarditis that can lead to acute death or chronic heart failure symptoms with death by six months of age.[1, 11, 23]

Once infected, CPV localizes to the lymphoid tissue of the oropharanyx where it replicates before entering the bloodstream. Viremia will usually occur two to three days post-infection, and is manifested by a slight anorexia and fever. Dogs recover from this stage briefly before progressing into clinical enteritis, typically between days five and six post-infection.[11] Clinical signs of enteritis include anorexia, vomiting, diarrhea and severe dehydration. Severity of signs is related to age, stress level (other disease processes, weaning), immune status and presence of intestinal parasites. These signs will usually pinnacle around day six to seven post-infection.[11]

CPV infects and destroys the epithelial cells of the intestinal crypts. These cells are responsible for the regeneration and differentiation into the villous epithelial cells. Therefore, without the intestinal crypt cells, there is marked villous blunting, atrophy and even necrosis, as well as malabsorption of nutrients with resultant diarrhea. Intestinal inflammation accompanies this destruction and can lead to the breakdown of the gut-blood barrier. Translocation of bacteria from the gut lumen into the bloodstream, along

with an impaired immune response, can result in bacteremia. Bacteria are most often gram negative, releasing endotoxin upon their destruction. Endotoxemia can then lead to systemic inflammatory response syndrome through the activation of various cytokine mediators, progressing into multiple organ dysfunction syndrome and death.

Leukopenia is considered a characteristic and often diagnostic quality of CPV infection. However, this finding is reported to be evident in as few as 33% of cases when diarrhea first developes.[6] Most pronounced of the decreased white blood cells is the neutrophil portion. This neutropenia is postulated to be due to a combination of significant intestinal tract inflammation acting as a sink for the cells, rapid depletion of bone marrow stores, and infection of the granulocyte precursors in the bone marrow. Interestingly, it appears that the CPV spares the early hematopoietic precursors and destroys mainly the later stages. Neutrophil nadir was found to coincide with the days when clinical signs were at their worst at roughly day seven to eight post-infection. In addition, neutrophil recovery coincides with clinical recovery at approximately days eight through 12.[11] A rebound neutrophilia is often observed as granulopoiesis is stimulated with a decreasing peripheral consumption of mature neutrophils and a decreasing central destruction of their progenitor cells. Hypoproteinemia, in particular hypoalbuminemia, is another common clinicopathologic abnormality associated with CPV enteritis. This is a result of a combination of factors, including intestinal loss, decreased synthesis as a negative acute phase protein and decreased nutritional intake. Other laboratory abnormalities vary, and can include anemia, azotemia, or increased liver enzymes.

Maternal antibodies can offer protection to pups from CPV for the first few weeks of life. The period of protection varies, depending on maternal antibody level, amount of

colostrum ingested, and size of the litter. The titer of the pup will equal approximately 50-60% of the bitch's titer at whelping, meaning that proper vaccination of the mother is essential to conferring protection to her pups.[23] Maternal antibodies have a half-life of around 10 days in the pup, and can interfere with proper immune response to vaccination.[23] The primary cause for vaccine failure is interference by maternal antibodies. The high titer (higher amount of virus per dose), low passage (fewer times virus grown on tissue culture) vaccines tend to be more effective despite maternal antibody levels.[23] A schedule of vaccination starting at six weeks old with boosters every three weeks until 12-16 weeks old is the current recommendation to avoid interference and provide proper protection to the pup. Proper vaccination is highly recommended, as unvaccinated dogs have been found to be 12.7 times more likely to develop CPV enteritis when compared to vaccinated dogs.[24] Local antibody activity is responsible for binding intestinal CPV to prevent further virus shedding in the feces. Shedding will typically occur until between days three and 10 post infection. However, it is the systemic humoral immune response or the circulating antibodies that confer protection as they inactivate the virus in the blood before it has a chance to invade intestinal, lymphoid or bone marrow cells.

Treatment of CPV consists principally of supportive care. Maintaining hydration is the staple of this supportive care. Balanced electrolyte solutions supplemented as needed with potassium and dextrose are the primary fluid type of choice. Colloidal solutions can be used when indicated to support colloidal osmotic pressure. Antibiotic use is an area where opinions vary, although their use is generally accepted once the neutrophil count falls below 1500 cells/ul. Antibiotics targeting enteric bacteria are

recommended, such as a combination of ampicillin and enrofloxacin. A lower dose of 5mg/kg q12 hours and use for a limited time (i.e. less than 5 days if possible) are precautions taken to help avoid the side effect of cartilage deformities in these young dogs. Antiemetics such as a constant rate infusion of metaclopramide and/or chlorpromazine are frequently used to help control the vomiting associated with the disease. The concept of starvation to rest the gut is being challenged lately. A recent study found that dogs fed through a nasoesophageal tube soon after admission and through vomiting episodes showed a significantly more rapid clinical improvement without major adverse side effects.[10] These dogs were found to have a quicker recovery of appetite and normal attitude as well as recovery from vomiting and diarrhea when compared to the food restricted group.

Several other potential treatments have been tested, often with conflicting or disappointing results. Treatment with equine LPS endotoxin antiserum has shown varying results. One source reports that its use decreased mortality from 48% to 17%.[12] Another study found that its use was associated with an increased risk of death in puppies less than 16 weeks.[7] Recombinant human granulocyte colony stimulating factor (rhG-CSF) has been shown to help reverse neutropenia associated with radiation therapy and drugs as well as cyclic neutropenia in dogs. However, its use to treat the neutropenia of CPV infection has not shown significant benefit in recovery time or neutrophil response.[8, 9] Bactericidal/permeability-increasing protein (BPI) is an enzyme located in the granules of polymorphonuclear cells. It is cytotoxic to gram negative bacteria by increasing membrane permeability, and will also bind to free LPS to block its effects. With the finding that 82% of CPV enteritis cases have endotoxemia, and that a

relationship exists between increasing detectable tumor necrosis factor activity and mortality,[6] it was postulated that administration of a recombinant BPI ($rBPI_{21}$) would help improve survival. Unfortunately, $rBPI_{21}$ treatment was not found to have a significant effect on outcome, hospitalization time or plasma endotoxin levels in one study.[2] Interferons have antiviral effects that vary depending on dose, species and virus type. A recombinant form of feline interferon omega (rFeIFN) has shown promise in reducing severity of disease and mortality,[13, 14] warranting further investigation

It is not necessarily the infection of the rapidly dividing cells and resultant gastroenteritis that causes the high morbidity and mortality associated with CPV disease. Gnotobiotic dogs infected with CPV were found to have only mild clinical signs.[6] This is highly suggestive that it is the bacterial translocation and resultant septicemia that is significantly related to the morbidity and mortality of CPV enteritis. Endotoxemia with subsequent systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) are associated with a high mortality in and of themselves. E. coli has been cultured from the lungs and liver in up to 90% of dogs that have died from CPV,[22] illustrating the high potential for bacteremia to develop as well as its devastating consequences.

Oseltamivir is a drug that was designed to inhibit the human influenza A and B viruses through inhibition of neuraminidase. Hemagglutinin (HA) and neuraminidase (NA) are two viral surface proteins that interact with receptors containing terminal neuraminic acid residues.[15] The purpose of the HA is to mediate viral attachment to the host cell membranes to allow entry and subsequent replication. NA has multiple functions that allow for the replication of the virus as well as enhancing its virulence.

First, NA cleaves the sialic acid residue (N-acetyl-neuraminic acid is synonomous with sialic acid) of the cellular receptor binding newly formed virons.[20] This frees the virons from the host cell, and allows their systemic release. Secondly, NA prevents the aggregation of the virons, allowing more effective systemic distribution with subsequent cellular infectivity.[20] Thirdly, in order to come in contact with and invade respiratory epithelial cells, the influenza virus must first penetrate through the protective mucin layer covering these cells. This mucin contains abundant sialic acid residues. NA cleaves these residues, facilitating the viral infiltration through mucin to the epithelial cells which the virus then invades and replicates within.[20] Inhibition of NA will result in a direct antiviral effect in viruses, such as the influenza virus, that contain the cell surface protein NA. These effects include decreased replication, decreased viral release, increased viral aggregation and decreased dissemination. In viruses that lack NA receptors such as CPV, NA inhibition may prevent viral migration through mucin and subsequent invasion of respiratory epithelial or gastrointestinal epithelial cells.

The above listed functions are accepted and proven effects of NA. In addition, there currently are hypothesized effects of NA on the immune system itself. Secretory IgA provides the body's primary immunologic defense of mucosal surfaces such as the respiratory or the gut epithelium. Under normal circumstances, sialic acid residues are covalently bound to the hinge region of the IgA molecule. Removal of these residues converts IgA into an appropriate ligand for the liver asialoglycoprotein receptor, which effectively removes IgA from circulation and results in its clearance.[20] Working on the unproven assumption that there is an equilibrium between circulating and mucosal IgA, the loss of IgA mediated by NA would result in impaired mucosal defenses. The IgA of

mucosal surfaces is secreted by B cells under the influence of gamma-delta T cells. Both of these cells contain sialic acid moieties on their surfaces.[20] If cleaved by NA, the lymphocyte homing would be altered from the mucosa to the bone marrow, resulting in a localized immunosuppression for that mucosal surface. It is also believed that NA itself may allow the increased production of certain cytokines,[15] resulting in a pro-inflammatory effect. Thus, inhibition of neuraminidase has the potential to improve mucosal immunity by increasing the amount of mucosal IgA, decreasing the clearance of circulating IgA, allowing normal function of the B and T cells and decreasing the cytokine release associated with the proinflammatory response. All of these effects could be beneficial to a dog with severe gastrointestinal inflammation secondary to CPV infection.

Effects of the neuraminidase inhibitors beyond direct antiviral effects have been appreciated in multiple studies. Bacterial complications following influenza virus infection in children and adults are common. The incidence of acute otitis media complicating influenza infection in children ranges between 21% to over 50%.[12] Use of oseltamivir has been reported to reduce the incidence of otitis media by 44% (children 1-12 years) and 56% (children 1-5 years).[21] In adults, secondary complications were reduced from 15% in the placebo group to 7% in the oseltamivir group in one study.[25] Secondary bacterial pneumonias are responsible for an estimated 25% of influenza-related deaths[19] in adults. *Streptococcus pneumoniae* is a bacteria commonly associated with pneumonia secondary to influenza infection. A lethal synergism between the influenza virus and S. pneumonia has been established,[17] presumably accounting for the high morbidity and mortality associated with the combination. It has been shown that the

influenza virus promotes adherence of *S. pneumonia* to respiratory epithelium.[17, 18] Viral NA cleaves the sialic acid residues on the host respiratory epithelial cell, revealing cryptic binding sites for the bacteria,[17, 18] thus facilitating colonization. Oseltamivir reverses this effect in vitro.[17]

In a mouse model of influenza with secondary pneumococcal pneumonia, oseltamivir use alone increased survival from 0% to 75%.[18] This study also found that oseltamivir use in addition to proper antibiotics resulted in 100% survival, compared to 0% in mice treated with antibiotics alone. Prophylactic use of oseltamivir resulted in significantly less weight loss than placebo treated mice (prophylactic use, 5%; placebo, 25%).[18] Prophylactic as well as delayed treatment use decreased the development of pneumonia, increased survival and slowed the development and progression of disease in those animals that did acquire pneumonia.[18] In adults, oseltamivir use was found to decrease the incidence of lower respiratory tract complications (LRTC) in influenza patients overall by 55%.[16] Patients considered at risk for secondary complications showed a decrease in LRTC and antibiotic use by 34%, and in otherwise healthy patients by 67%.[16] In these studies, the beneficial effect of oseltamivir in preventing secondary bacterial infections is most likely the result of NA inhibition preventing bacterial and viral penetration of the mucin layer to allow bacteria to bind to epithelial cells.

Oseltamivir is labeled for use in children over 1 year old; use in children less than 1 year is discouraged based on fatality seen in animal toxicology studies in seven day old rats with high doses of drug.[21] Although the etiology of such adverse effects has not been specifically identified, there is concern over lack of a fully developed blood brain barrier. Side effects in children over 1 year are minimal, with vomiting being the only significant

effect versus placebo.[21] Similarly, nausea (12% vs 4%) and vomiting (10% versus 3%) were found to be significant side effects over placebo in adults that subsided after the first few days.[27] These effects can be diminished with administration of a small amount of food along with the drug. Studies in adult humans have shown an 80% bioavailability of oseltamivir after oral dosing.[21]

STATEMENT OF RESEARCH OBJECTIVES

This study was designed to investigate the influence of oseltamivir when added to standard treatment on the disease process of CPV enteritis, as well as to monitor for significant adverse side effects associated with its use. Our hypothesis was that oseltamivir would help to ameliorate the disease morbidity and mortality associated with naturally occurring CPV enteritis, thereby decreasing hospitalization time, the need for colloid and other adjunctive therapies, and thus the cost associated with treatment.

MATERIALS AND METHODS

Study Design

The present study was a prospective, randomized, placebo-controlled, blinded clinical trial of the effects of oseltamivir in dogs with naturally occurring parvoviral enteritis. Cases of CPV enteritis were recruited from the local area and animal shelter between April 2005 and August 2006. A financial incentive was offered to the owners or agent to allow their dogs to participate in the study. Inclusion criteria was simply a positive CPV fecal antigen test, presence of appropriate clinical signs (vomiting, diarrhea, lethargy, anorexia), and lack of any treatment initiated prior to presentation. Informed

consent was received from all owners or agents prior to any treatment initiation at the Auburn University Veterinary Teaching Hospital. In addition, all procedures were reviewed and approved by the Animal Care and Use Committee of Auburn University. Dogs were excluded if they lacked a positive fecal antigen test for CPV, if treatment had been administered prior to presentation, or if consent was not granted. Based on these criteria, only one dog that was eligible during the trial period was not included due to lack of owner consent. All dogs that entered the study completed the trial.

Randomization was achieved via assignment of numbers with predetermined treatment designation in blocks of 5. Assignment was allocated by hospital personnel not directly involved in the trial, and these assignments were uncovered to the investigators only at the conclusion of the study. Power analysis was not performed as part of the design of the study, but post-hoc analysis revealed that 47 dogs would be needed in each group to give the study a power of 0.80 at a significance level of 0.05.

Standard Treatment

A treatment protocol was designed to standardize therapy between the two groups with the exception of oseltamivir administration to the treatment group and a placebo to the control group. No deviations were made from this protocol for any of the patients. An intravenous catheter was placed in all dogs, either a peripheral cephalic catheter or central venous catheter in a jugular vein. Intravenous fluids consisting of a balanced electrolyte solution [b, c] were administered at an initial rate to replace an estimated dehydration deficit over a minimum of 2 hours and a maximum of 4 hours. In the case were it was determined that shock was present, the same intravenous fluids were bolused at a dose of 10-30ml/kg over approximately 20 minutes. Boluses were repeated until it

was assessed that the state of shock had been resolved, and the remaining fluid deficit estimated to replace dehydration was administered as above. No patient required the administration of colloidal therapy to treat a state of shock. Assessment of hydration and perfusion status, as well as fluid rates were decided upon by a single investigator (MRS) for all dogs. After rehydration, the hydration status was reassessed via physical parameters and the fluid rate adjusted as deemed appropriate. Fluids were supplemented with potassium chloride[d] and/or dextrose[e] as needed based on laboratory results.

All dogs received intravenous (IV) antibiotics. Ampicillin[f] was administered at 22mg/kg (11mg/lb) IV every 8 hours and enrofloxacin[g] at 5mg/kg (2.5mg/lb) IV every 12 hours. A metoclopromide[h] constant rate infusion was also initiated in all patients at a rate of 1-2mg/kg/day (0.5-1mg/lb/day). If vomiting persisted at a rate of more than twice per 12 hour period despite a maximum rate of metoclopramide, chlorpromazine[i] was added at 0.5mg/kg (0.25mg/lb) subcutaneously every 8 hours. Pyrantel pamoate[j] (10mg/kg [5mg/lb] per os) was administered to all dogs within the first 3 days of arrival to eradicate intestinal parasites. If the total solids by refractometery fell below 3.5g/dl, Hetastarch 6%[k] was infused at a rate of 10-20ml/kg/day (5-10ml/lb). If anemia, defined as a PCV of less than or equal to 20%, was present, a fresh whole blood transfusion from an available donor was given at 10-20ml/kg (5-10ml/lb) over 4 hours. Packed red blood cell availability was very limited at the time of the first required transfusion. Fresh whole blood was collected from a donor dog and administered in its place. In order to minimize differences in treatment between dogs, when the second transfusion became necessary, although packed red cells were available, fresh whole blood from the previous donor was again administered. If vomiting episodes were less than 4 per 12 hour period, water and a

bland diet[l] were offered. If either appeared to induce nausea, they were immediately removed from the animal. Voluntary eating was allowed through mild vomiting but food was not forced upon any dog. True client cost for the treatment administered to the dogs was not calculated; this figure can only be inferred from the intensity of treatments (additional antiemetics, colloid or transfusion therapy, increased length of hospitalization) required for each dog between the groups.

Discontinuation of intravenous fluids was at the discretion of a single investigator (MRS). This was accomplished primarily when the animal was no longer vomiting and consistently eating and drinking sufficiently to maintain hydration. Once fluids were discontinued, all patients were monitored for at least 12 hours before discharge from the hospital in order to ensure no relapse of clinical signs. The day of discharge was assigned as the day when it was deemed the dog was healthy enough to be discharged. If the dog stayed in the hospital for non-medical reasons, these extra days were not included when figuring length of hospitalization. In those animals that did not survive, the day of death was considered an endpoint equivalent to the day of discharge for the purpose of data analysis. Post-mortem exams were performed on all non-surviving dogs.

Oseltamivir Treatment

Dogs assigned to the treatment group received oseltamivir at 2mg/kg (1mg/lb) per os every 12 hours. An equivalent volume of a placebo, consisting of a standard suspension agent with color additive, was administered per os every 12 hours to dogs in the control group. Prior experience with oseltamivir by the authors has shown that dogs often react to its taste and frequently vomit shortly after administration. Dilution of the oseltamivir with water (1:1) prior to administration seemed to decrease these reactions;

therefore, in this study, both oseltamivir and placebo were diluted out as above to lessen the risk of adverse reaction to the oseltamivir. In addition, in dogs receiving chlorpromazine for protracted vomiting, it was attempted to dose this medication 30-60 minutes before administration of the oseltamivir or placebo to minimize the risk of vomiting either suspension as often as coincidental timing of the drugs allowed (typically for the morning treatment). Given its propensity for gastric side effects (vomiting, nausea),[15] if significant adverse effects were associated with the administration of oseltamivir to these patients despite these precautions, it should have been reflected in the clinical scoring system employed.

Monitoring/Data Acquisition

Historical data, including previous vaccination against CPV and the duration of clinical signs as noted by the owner or caretaker (rounded to the closest 12 hour period of time), was obtained when this information was available at study entry. Signalment for each dog was also recorded, as was its estimated percent dehydration on entry. Baseline bloodwork was evaluated for all dogs on entry, consisting of a complete blood count (CBC), a spun packed cell volume (PCV), total solids (TS) via refractometery, serum electrolyte concentrations (sodium, potassium and chloride), blood glucose concentration and blood lactate concentration[m]. These values were monitored daily for all dogs. For white blood cell values, the following variables were evaluated: initial total white blood cell (WBC), neutrophil (neut) and lymphocyte (lymph) counts, the absolute nadir value and the day of hospitalization (with day of presentation being day 1) on which the nadir occurred for each of the WBC, neutrophil and lymphocyte counts, and the number of days for which the counts for each value were significantly decreased, as defined by a

WBC less than 3,000 cells/ul, neutrophils less than 2,000 cells/ul and lymphocytes less than 1,000 cells/ul. Body weight was recorded twice daily, with the same scale used to monitor each dog during their stay for consistency. In addition, vital parameters (heart rate, respiratory rate, rectal temperature, mucous membrane color and capillary refill time) were assessed a minimum of twice daily, as were hydration status and mentation. Days on which dogs demonstrated SIRS criteria were also calculated as a percentage of days of their total stay (days with positive SIRS criteria/total days in hospital x 100%). SIRS was defined as to presence of at least 2 of the following 4 criteria: 1) temperature greater than 102.5°F (39.2°C) or less than 100.0°F (37.8°C), 2)heart rate greater than 140 beats per minute (bpm), 3) respiratory rate greater than 40/minute or 4) total white blood cell count greater than 19,000 cells/ul or less than 6,000 cells /ul.

Clinical Scoring System

A previously published clinical scoring system[10] was used to evaluate 4 clinical attributes of each patient: attitude, appetite, vomiting and feces. A score of 0 represented a clinically normal parameter, with increasing severity of signs as the score increased up to a maximum of 3 for each variable (Table 1). Scores were assigned twice daily, to encompass the previous 12 hour period, and were assigned by the same investigator for all dogs (MRS). The clinical scores were totaled for each dog per day for each of the 4 categories (attitude, appetite, vomit, feces), with a possible score ranging from 0 (normal) to 6 (most severely affected) per 24 hour period. This total consisted of the combined scores (ranging from 0 to 3) for each 12 hour periods for that day, assigned to each category. In addition, a cumulative score consisting of a total across all 4 categories was

calculated for each dog per day, with a possible score ranging from 0 (normal) to 24 (most severely affected).

STATISTICAL ANALYSIS

The Shapiro-Wilks test was used to evaluate the distribution of continuous variables. Median (minimum, maximum) was used to describe continuous variables not normally distributed and mean (+/- standard deviation [SD]) was used to describe normally distributed variables. The Wilcoxon ranksum test was used to compare not normally distributed continuous variables while the t test was used for normally distributed variables. Categorical variables were described using percent and the Fisher's exact test was used to compare these variables between the treatment and control groups. For all comparisons, a p-value <0.05 was considered significant. All statistical analyses were performed using a statistical software program[n].

RESULTS

A total of 35 dogs were evaluated, with 19 in the treatment group receiving oseltamivir and 16 in the control group receiving a placebo. There were 3 shelter dogs included in the study, all of which were in the control group. Most dogs (30/35) were of the mixed-breed variety, with the purebreds consisting of 2 American pit bull terriers and 1 each of dachshund, beagle, and Labrador retriever. No statistically significant differences were found between groups in the baseline characteristics of age, sex, vaccination status or duration of clinical signs prior to presentation. The median age of dogs in the control group was 14 (8, 36) weeks, while that of the treatment group was 12

(8, 44) weeks (p=0.50). There were 21 (60%) female dogs, with 10 (48%) randomized to the control group and 11 (52%) randomized to the treatment group. Alternatively, there were 14 (40%) male dogs, with 6 (43%) randomized to the control group and 8 (57%) to the treatment group (p=1.0). All dogs were sexually intact. Vaccination status was known for 10/16 (62%) dogs in the control group and 13/19 (68%) dogs in the treatment group. Of these, 5 (50%) dogs in the control group had received at least one vaccination against CPV, whereas 6 (46%) in the treatment group had been vaccinated at least once against CPV (p=1.0). The duration of clinical signs prior to presentation was known for 15/16 (94%) control dogs and 16/19 (84%) treatment dogs. Mean days sick for the control group was 1.4 (+/-0.9) days, while that for the treatment group was 1.8 (+/-1.0) days (p=0.31).

There was no significant difference in the degree of estimated dehydration at entry of dogs between groups. The control dogs were estimated to have a mean dehydration deficit of 6.3% (+/-1.54%), while the treatment dogs were estimated at 6.9% (+/-1.7%) (p=0.35). In addition, there was no statistical difference amongst the weights at entry or the weights at discharge between the two groups. Dogs in the control group had a median entry weight of 6.7kg (14.7lb) (1.8, 28.2kg [3.96, 62lb]), and those of the treatment group 4kg (8.8lb) (1.6, 25kg [3.5, 55lb]) (p=0.21). At discharge, the median weight of control dogs was 6.5kg (14.3lb) (1.8, 27.3kg [3.96, 60lb]) and that of the treatment group was 4.4kg (9.7lb) (1.6, 28.6kg [3.5, 63lb]) (p=0.42). However, a significant difference was found in the weight change from entry until discharge. Dogs in the control group experienced a median change of -0.21kg (-0.46lb) (-2.8, 0.5kg [-6.2, 1.1lb]), while those in the treatment group had a median gain of 0.07kg (0.15lb) (-1,

3.6kg [-2.9, 7.9lb]) (p=0.01). This correlates also to a significant difference in the percentage of change in body weight ([discharge weight – entry weight / entry weight] * 100%) (Figure 1). Dogs in the control group had a mean change of -4.5% (+/- 6.9%), and those in the treatment group a mean change of +2.6% (+/- 7.1%) (p=0.006). When this analysis was repeated for survivors only, the results were still found to be significant. The control dogs contained all 3 non-survivors, and the new calculation without these dogs resulted in a median percent weight change of -3.9% (-2.8, 0.5), compared to the median percent weight change of 1.3% (-1, 3.6) for the treatment dogs (p=0.012).

The percentage of days in the hospital that SIRS criteria were met was calculated for each dog. There was no significant difference between groups, with control dogs meeting SIRS criteria a mean of 52% of days, versus 54% for treatment dogs (p=0.91).

No difference was found in the duration of hospitalization between the two groups. Dogs in the control group had a mean stay of 5.9 (+/- 2.6) days, and those of the treatment group 6.0 (+/- 2.3) days (p=1.0). Colloid therapy was not required often, as the median number of days on colloids for the control group was 0 (0, 3) days and also 0 (0, 5) days for the treatment group (p=0.5). None of the 16 dogs in the control group received a blood transfusion, while 2/19 (10%) dogs in the treatment group did (p=0.5). The addition of chlorpromazine was necessary in 10/19 (53%) dogs in the treatment group, and 9/16 (56%) dogs in the control group. Dogs in the treatment group that did receive chlorpromazine did so an average of 21% of their days in the hospital, while the dogs requiring it in the control group were given chlorpromazine an average of 30% of their days in the hospital. The overall survival rate was 91% (32/35). Three dogs died, all from the control group, giving this group a survival rate of 81% (13/16). One animal

was euthanized after severe progression of clinical signs despite treatment, and it was deemed that the dog was suffering and would not recover, while the other 2 suffered natural deaths. Survival in the treatment group was 100% (19/19). However, this difference was not found to be significant (p=0.09).

Post mortem examinations were performed on all non-surviving dogs. Findings were consistent with a diagnosis of CPV enteritis. All dogs had diffuse, severe, necrotizing enteritis. Bone marrow was examined in 2/3 dogs, both showing sections of moderate hypocellularity. While 2 dogs were noted to have diffuse congestion and edema in their lungs, the third dog was found to have a mild interstitial pneumonia.

The white blood cell values that were evaluated (initial counts for WBC, neutrophils and lymphocytes, the nadir values and day of nadir, and significant decreases in counts for WBC, neutrophils and lymphocytes), were compared between groups. No significant differences were found for any of these values between groups. Although it was not found to be significant, it was noted that the treatment dogs had a very slightly lower average WBC (7,500 cells/ul) and neutrophil count (6,220 cells/ul) at presentation than the control dogs (8,540 cells/ul WBC and 6,830 cells/ul neutrophils). Both groups experienced a decline in numbers with an average nadir of WBC at 2,680 and 2,810 cells/ul for the treatment and control groups, respectively, and 1,160 and 1,240 cells/ul for the neutrophils for the treatment and control groups, respectively. While by Day 5 the control dogs had rebounded their WBC (4,580 cells/ul) and neutrophil counts (2,270 cells/ul), the treatment dogs showed a slightly higher value for each at the same time point (6,920 cells/ul WBC and 4,310 cells/ul neutrophils). Seven of the 16 (44%) control dogs had a total WBC nadir occur at less than 100 cells/ul. Two of these 7 (29%) dogs

did not survive (1 died, 1 euthanized). In the treatment group, 8/19 (42%) dogs had their total WBC nadir occur at less than 100 cells/ul. None of these dogs died.

Comparison of significant decreases in white blood cells, neutrophils and lymphocytes on initial presentation and on Day 4 of hospitalization is shown in Figure 2. Based on the expected timeline of disease progression as previously described,[11] Day 4 was chosen for this comparison to represent a period in time that should have encompassed neutrophil and clinical recovery for most dogs. In general, a higher percentage of treatment dogs versus control dogs fulfilled the criteria for significant decreases in initial white blood cells and neutrophil counts. However, by Day 4, more control dogs were significantly affected.

When the clinical scores were compared day by day for each category as well as the cumulative total for that day, no significant differences were found between groups (Figures 3) with the exception of Day 6 for the appetite score ($p=0.02$). There was also a difference for attitude on Day 6, but this was not significant ($p=0.086$). Looking at the scores for dogs in each group as compared to their white blood cell counts on the same day (Table 2), it can be seen that the treatment dogs did have mildly improved scores across the board on Days 4 and 5 while also having a quicker rebound of WBC and neutrophil counts. Days 4 and 5 were chosen for examination for the same reason as stated above.

DISCUSSION

Findings

While the use of oseltamivir in addition to standard therapy for naturally occurring CPV enteritis did not exhibit a significant effect to help decrease hospitalization time, additional treatments needed or disease morbidity as determined by a clinical scoring system, it was shown to be associated with a significant increase in weight change versus control dogs from entry until discharge. The control dogs tended to lose weight, while the treatment dogs gained. This finding was not affected by the degree of dehydration at presentation, as it was found that there was no significant difference between the two groups in this variable.

The importance and implications of this finding are unknown at this time. Other studies have shown that a significant change in weight in study subjects is also associated with an improved outcome. In one study using a mouse model of human influenza infection with secondary bacterial pneumonia, it was found that prophylactic treatment (i.e. dosage begun 4 hours prior to viral infection) with oseltamivir resulted in an average loss of only 5% of body weight in the mice, compared to an average of 25% loss in the placebo mice.[18] This study also showed that all mice in the prophylactic oseltamivir group survived the viral and bacterial challenge, versus none of the mice in the placebo group. A second study was investigating the effect of early enteral nutrition (EEN) on dogs with parvoviral enteritis.[10] In this study, it was found that dogs in the EEN group had a significant increase in weight gain from entry on all days of the study, while the conventional group had no significant change in weight. In addition, dogs in the EEN group showed a more rapid clinical improvement, based on normalization of clinical

scores, than did the conventional group. While the difference was not significant, it was also found that the 2 dogs that did not survive were both from the conventional group, giving it a survival rate of 87% compared to 100% for the EEN group. These survival statistics are very similar to those found in the present study. These results suggest that the significant change in weight associated with oseltamivir use in this study could imply that more significant beneficial effects on survival and/or disease recovery could also be a plausible effect of the oseltamivir that was not brought out here.

The use of oseltamivir did not appear to be associated with any significant adverse effects. The main side effects reported in humans are gastrointestinal effects apparently due to direct local irritation of the gastric mucosa.[15] In the experience of the authors, dogs will also often react to the taste of the oseltamivir suspension and nausea and vomiting can be encountered. Dilution with water just prior to administration appears to minimize these effects. This practice was utilized in this study in an effort to not only avoid uncovering group assignment and thereby instituting a bias, but also in an effort to keep the clinical scores an accurate representation of the disease process in the animal and not obscure these scores with drug reaction.

Analysis of the clinical scores, specifically vomiting and appetite, did not show any difference between the treatment and control groups. This would tend to support the lack of any significant adverse effects of oseltamivir administration versus placebo in this patient population. The possibility that oseltamivir did have an effect to improve the clinical effects of CPV enteritis in the treatment dogs, but itself caused increased vomiting and nausea as a side effect of the drug and therefore concealed any benefit evident by analysis of the clinical scores does exist. However, subjective observation

was that administration of the oral medications (oseltamivir or placebo) was not associated with initiating increased nausea or vomiting directly afterwards. This supports the interpretation of results indicating that side effects of oseltamivir were minimal, as were beneficial effects on decreasing disease morbidity.

The intensity of treatment required and the expected cost of treatment were inferred based on additional therapy, such as colloid infusion or blood transfusions, as well as prolonged hospitalization times, which were needed. Colloids were not frequently used, in contrast to a previous report.[26] Specifications for the institution of colloid therapy were not elucidated in the previous study. Protocol in this study required significant decrease in total solids to 3.5g/dl. Much more modest decreases, often around 4.0 to 4.5g/dl, are frequently used in the clinic setting as indication for treatment with colloids. A higher cutoff value as a trigger for colloid infusion likely would have increased the incidence of its use in this study. Whether this increased use would translate into a difference in usage between groups is unknown, but seems unlikely based on the findings of a previous study showing no difference in albumin concentrations between an EEN group and a conventional therapy group.[10] Blood transfusions were also rarely indicated. The 2 cases that did require a transfusion received fresh whole blood, rather than packed red blood cells. Packed red blood cell availability was very limited at the time of the first required transfusion. Fresh whole blood was collected from a donor dog and administered in its place. In order to minimize differences in treatment between dogs, when the second transfusion became necessary, although packed red blood cells were available, fresh whole blood from the previous donor was again administered. The total volume of crystalloids as well as colloids administered between the two groups may

have been helpful to evaluate. Dogs experiencing greater fluid loss from vomiting and/or diarrhea as well as having less voluntary intake per os would have a greater intravenous fluid need. This would correlate with an increased severity of clinical signs and manifestation of the disease process. Unfortunately, the collection of this data had significant gaps or was outright missing from certain dogs due to recording error or technical difficulties. Therefore this value was not analyzed.

The duration of clinical signs prior to presentation was not different between the groups. It has been reported that for human influenza viral infections, oseltamivir is most effective if started within the first 12 hours of clinical signs, with efficacy decreasing up to 48 hours.[24] For every 6 hour delay in starting oseltamivir, the duration of illness is predicted to increase by approximately 8%.[25] Whether this also holds true for its use in CPV enteritis is unknown. Because replication of CPV does not depend on neuraminidase, administration of oseltamivir in the early stages of infection is unlikely to diminish viral replication and dissemination as with the influenza virus, and thus a time-efficacy response is not expected. Rather, with the proposed mechanism against bacterial translocation, oseltamivir may have a greater impact when administered during the period of leukopenia and severe clinical signs. Further investigation is needed to expand on these speculations.

The treatment group may have been further along in their course of disease, despite the lack of difference in reported duration of clinical signs. This would be supported by the fact that this group, on average, had a slightly lower white blood cell and neutrophil counts at entry, as well as a somewhat quicker rebound after these numbers dropped. The clinical scores also showed the trend of quicker improvements in

the treatment group. With the inherent inaccuracies of estimation of duration of clinical signs by the owner or caretaker, it is plausible that the times reported at entry were not correct, and that the treatment group did include dogs at a more advanced stage of disease. Conversely, if the reported duration of signs was accurate, the seemingly quicker recovery of the treatment dogs could be attributed to a beneficial effect of oseltamivir.

A clinical scoring system was utilized in order to evaluate the subjective criteria of attitude and appetite, as well as to quantify the severity of vomiting and characterize the feces to allow for comparison across dogs. One investigator (MRS) had the responsibility of assigning scores to all dogs to minimize inter-observer variability. This investigator was also blinded to group assignment in order to minimize bias.
Values for each category and cumulative scores were compared between the 2 groups for each day, and although mild trends could be seen for lower scores in the treatment group, there were no significant differences. This could be attributed to the small sample size in this study, as well as the variability in the timeline of illness among dogs. Since dogs presented in all stages of their disease (i.e. clinical symptoms for less than 12 hours to up to 4 days), a straight out comparison of scores per day may not illustrate true differences and a larger group would be needed to further examine this effect. In addition, the clinical scoring system utilized is a very simple system, and as such, was relatively insensitive in its ability to differentiate between various stages of aberrancy in each of the clinical attributes. There was not much room to allow for representation of subtle yet clinically significant differences. A scoring system with a greater degree of stratification

between assigned values may allow for a greater sensitivity and a more accurate representation of the clinical status of the patient.

CPV is not reliant on neuraminidase for replication. However, anecdotal reports of the use of oseltamivir in dogs with CPV enteritis have claimed decreased morbidity and shortened recovery time in the treated dogs. It is speculated that the drug may inhibit bacterial translocation that subsequently leads to endotoxemia, sepsis, SIRS and death. Bacterial adherence and colonization of respiratory epithelial cells is potentiated in the presence of viral NA, and inhibited with NA blocking agents.[17-19] It is believed that the bacteria that commonly invade the lower respiratory tract express their own NA, thus enabling them to penetrate the protective mucin layer and infect the epithelial cells.[17] Although unproven, a similar mechanism may exist in the gastrointestinal tract. Oseltamivir may exert a beneficial effect by inhibiting NA on enteric bacteria, preventing their translocation across the gastrointestinal mucosal barrier. In CPV enteritis, the mucosal barrier is already impaired, allowing easier passage of bacteria. If bacterial NA plays a role similar to that in the lungs, the NA would cleave sialic acid residues on the gut epithelium, exposing receptor sites for bacterial adherence and further encouraging translocation. In addition, CPV suppresses the dog's immune system, both humoral and cell-mediated factors, allowing for systemic spread of bacteria and the resultant deleterious effects. Further studies are needed to accurately define the actual mechanism behind the observed anecdotal benefits of the use of oseltamivir to treat CPV enteritis.

Limitations

Limitations of this study do involve the concern of administration of an oral medication to a vomiting patient, and its variable systemic absorption in the face of a

diseased gastrointestinal tract. Gastric emptying times are quicker for liquid substances as compared to solids, with gastric emptying starting as soon as 10-30 minutes after administration. The oseltamivir suspension thus has the probability of starting to move through the gastrointestinal system and being absorbed before an episode of vomiting occurs. In addition, the act of vomiting is reported to only empty 40-50% of stomach contents. This would suggest that even with the presence of vomiting, it is reasonable to expect that at least a portion of the drug will remain in the system. Early safety studies of oseltamivir show that it has a bioavailability of 73% in healthy dogs, with detectable levels of the drug in plasma within approximately 30 minutes after oral administration.[27] Oseltamivir does require transformation to its active metabolite by esterases located within the liver, and to a certain degree, within the intestinal system.[27, 28] The importance of the intestinal system esterases is unknown. Due to this need for transformation, it is believed that oseltamivir effects are not due to a purely local action, but do require systemic distribution. The effect of a diseased gastrointestinal system such as that seen in CPV enteritis on the absorption, systemic distribution and transformation is unknown at this time and future pharmacokinetic studies, in this situation especially, are needed.

The reasoning behind any beneficial effect of oseltamivir in the treatment of CPV enteritis suggests that it helps decrease bacterial translocation and therefore the ensuing endotoxemia, SIRS and MODS that can develop. In this study, although certain physical and clinic pathologic parameters were monitored, there was no direct test for the presence of bacterial translocation or sepsis. SIRS criteria were evaluated, but this is a fairly crude assessment, especially considering the confounding factors. As the dogs were starting to

feel better, they would oftentimes become very excitable when being handled. This frequently resulted in an elevation of their heart rate to over 140 bpm, or their respiratory rate to greater than 40/min. The presence of these two variables would classify these healthy, excitable puppies as being positive for SIRS. In addition, the effect of the virus itself on the white blood cell count confounds the definition slightly. A WBC of less than 6,000 cells/ul can simply reflect destruction of progenitor cells in the bone marrow and is not necessarily associated with systemic inflammation. Other methods to evaluate for the presence of bacterial translocation, endotoxemia or SIRS would be more fruitful. Culture of mesenteric lymph nodes is considered the gold standard in human medicine and animal models for evaluation of bacterial translocation.[29] The feasibility of this procedure in this patient population (client-owned, live animals) and setting is questionable. Other methods, such as blood cultures or measurement of serum endotoxin levels or other inflammatory mediators may provide a more accessible method for differentiating animals in which bacterial translocation is present from those in which it is not. Further investigation would be needed before any true conclusions can be made.

CONCLUSIONS

CPV enteritis can be a devastating disease process. The financial constraints often encountered with treatment can be very frustrating given the treatable nature of this disease. Despite the anecdotal reports touting the success of oseltamivir to decrease the disease morbidity and mortality of CPV enteritis, scientific evidence of this was not found in this study. However, a significant difference in the change in body weight during hospitalization stay was established, as was the apparent safety of the drug in this

patient population. It is believed that, given the paucity of adverse side effects and the findings presented in this study, further investigation is warranted not only for its effects in CPV enteritis, but possibly any disease state in which bacterial translocation is a concern.

FOOTNOTES:

[a] Oseltamivir phosphate, Roche Laboratories Inc., Nutley, NJ.

[b] Lactated Ringer's Solution, Abbott Laboratories, North Chicago, IL.

[c] Normosol-R, Abbott Laboratories, North Chicago, IL.

[d] Potassium chloride, Phoenix Pharmaceutical Inc., St. Joseph, MO.

[e] Dextrose 50% solution, Veterinary Laboratories, Inc., Lenexa, Kansas.

[f] Ampicillin, Abraxis Pharmaceutical Products, Schaumburg, IL.

[g] Enrofloxacin injectable solution, 2.27%, Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS.

[h] Metoclopramide injection USP, SICOR Pharmaceuticals, Inc., Irvine, CA.

[i] Chlorpromazine HCl injection USP, Baxter, Deerfield, IL.

[j] Pyrantel pamoate 50mg/ml, Pfizer Animal Health, New York, NY.

[k] 6% Hetastarch, Hospira, Lake Forest, IL.

[l] Science Diet Canine I/D, Hill's Pet Nutrition Inc., Topeka, KS.

[m] Nova, Nova Biomedical, Waltham, MA.

[n] Stat 8.0 for Windows, College Station, TX

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Score	Attitude	Appetite	Vomiting	Feces
0	Normal	Normal	Absent	Well-formed or absent
1	Mild to moderate depression	Voluntarily eats small amounts	Mild; once per 12 hours	Soft or pasty feces
2	Severe depression	No interest in food	Moderate; 2-5 times per 12 hours	Watery diarrhea, non-bloody
3	Collapsed or moribund	Not offered	Severe; >6times per 12 hours	Watery, bloody diarrhea

Table 1: Clinical Scoring System. Scores for each category were assigned to each dog twice daily to encompass the previous 12 hour period.

	TREATMENT			CONTROL		
	INITIAL	DAY 4	DAY 5	INITIAL	DAY 4	DAY 5
WBC	7.5	4.97	6.92	8.54	4.09	4.58
NEUT	6.22	3.02	4.31	6.83	2.27	2.27
LYMPH	1.05	1.57	1.83	1.92	1.50	2.05
ATTITUDE	2	1	1	2	2	2
APPETITE	5	2	2	6	4	3
VOMIT	2	1	1	2	3	2
FECAL	3	2	2	2	3	3
CUMULATIVE	12	7	6	13	11	9

Table 2: Comparison of values between groups across time. Average values for WBC, neutrophils and lymphocytes (x 1000 cells/ul), and clinical scores at presentation and on Days 4 and 5 of hospitalization.

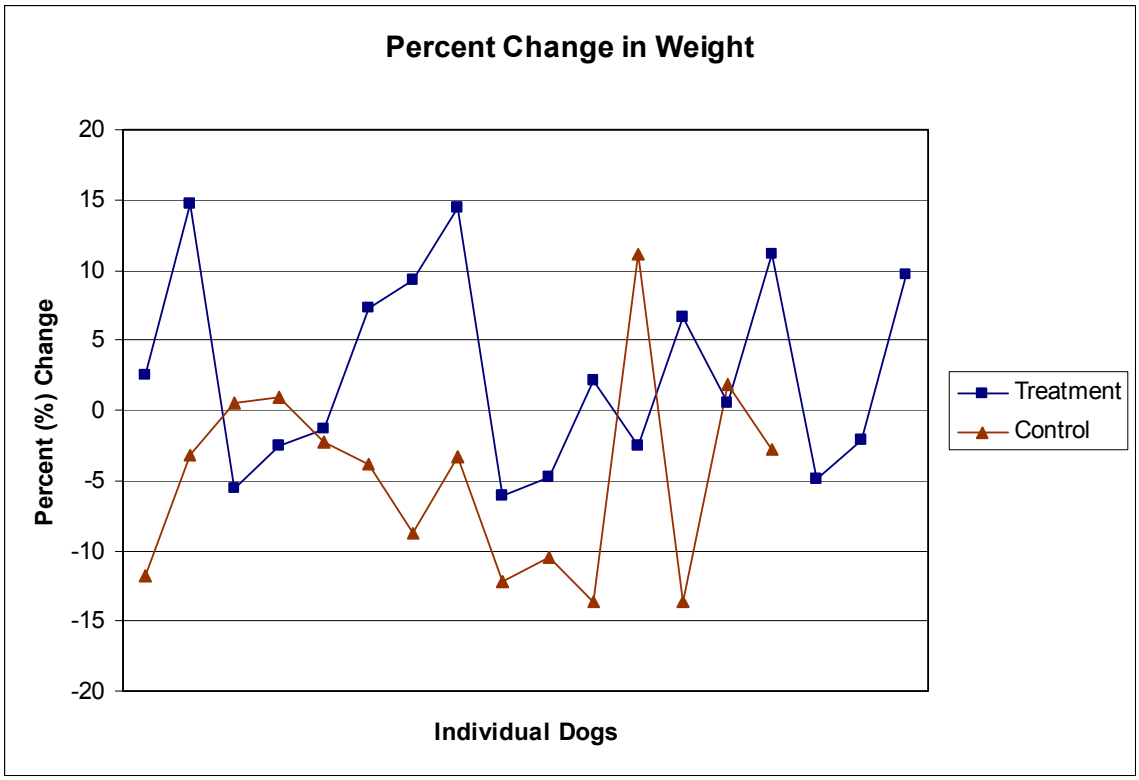

Figure 1: Percent weight change from entry until discharge for individual dogs. A significant difference (p=0.006) was found in the change in weight from entry until discharge between the control and treatment groups. The control group lost a mean of 4.5% (+/- 6.9%) of their body weight, and the treatment dogs gained a small amount with the mean change of +2.6% (+/- 7.1%) of their body weight. Percent weight change was calculated as: (discharge weight – entry weight) / entry weight x 100%.

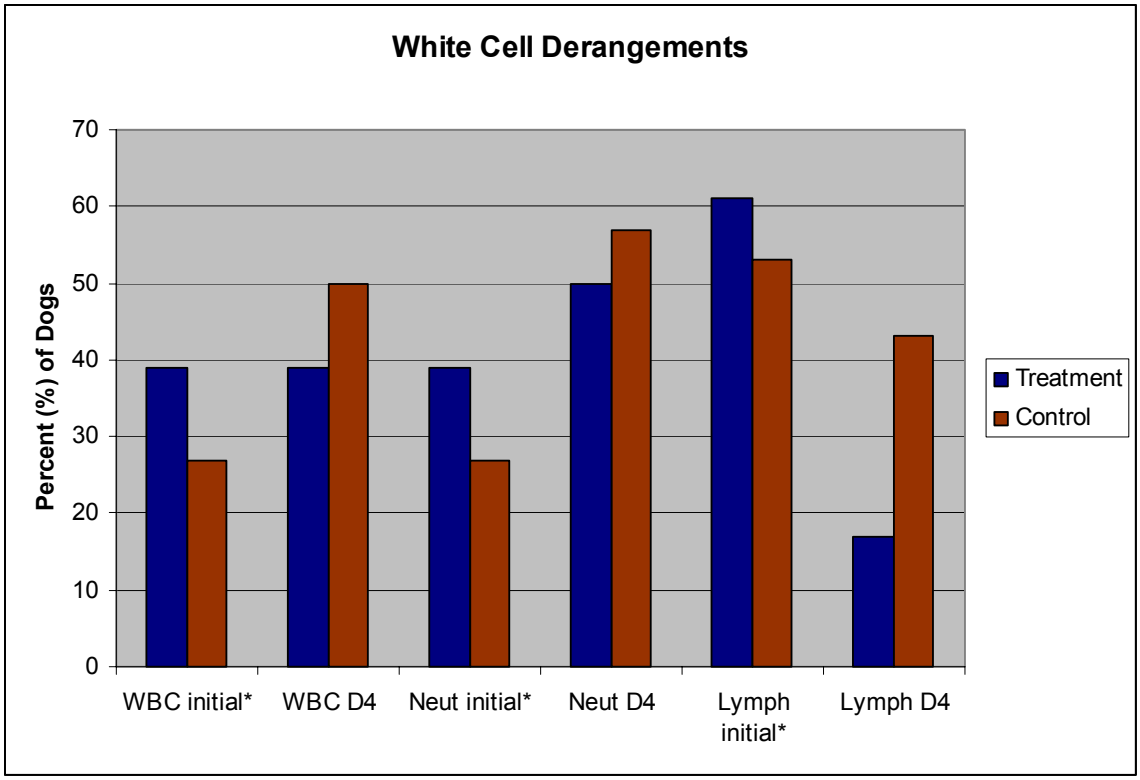

Figure 2: White blood cell value derangements. Percentage of dogs with significant decreases in white blood cells (WBC <3,000 cells/ul, neutrophils <2,000 cells/ul, lymphocytes <1,000 cells/ul) on presentation and on Day 4 (D4) of hospitalization.

* data missing from 1 dog from both treatment and control groups

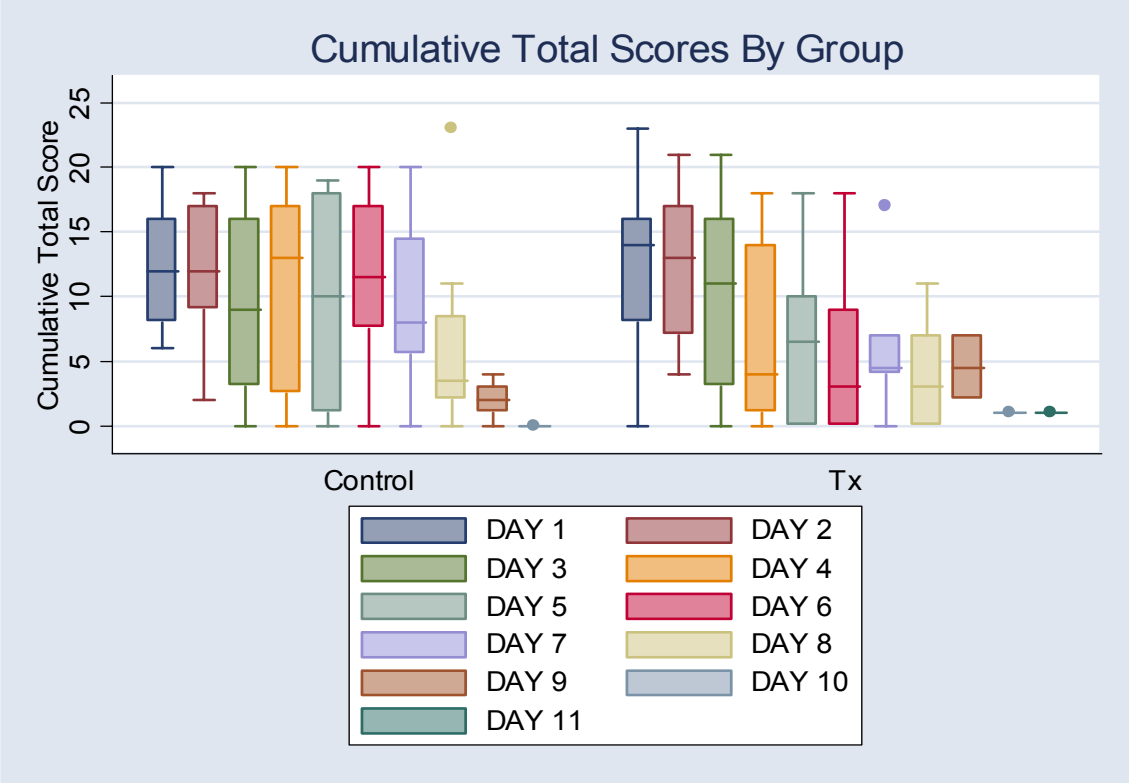

Figure 3: Box plot of cumulative total scores by day. A fair amount of overlap exists between the 2 groups, and no significant differences are present.