Evaluation of Immunosuppressants and Dietary Mechanisms in an Experimental Disease Model for Necrotic Enteritis

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ABSTRACT *Clostridium perfringens* (CP) is the etiologic agent of necrotic enteritis (NE). Clinical signs of this disease include depression, decreased appetite, diarrhea, and severe necrosis of the intestinal tract. Understanding the disease progression of NE has been difficult due to its complexity and the involvement of multiple factors (dietary components, immunosuppression, and mechanical irritation of the gut) that appear to contribute to this syndrome. In the present investigation, day-of-hatch broilers were fed a 55% wheat diet and randomly assigned to 1 of 8 groups. Treatments included positive control (CP challenge only), commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or the combination of CCV and CBDV, and an appropriate negative control for each (vaccinated and not challenged). Challenged treatment groups received 10^7 cfu of CP twice daily. When compared with controls, broilers in each treatment group had increased ($P \leq 0.05$) lesion scores, with mean scores of 1.05 and 2.05 in the CP and CBDV + CP treatments, respectively. When compared with controls, the incidence of CP increased ($P \leq 0.05$) in all treatment groups (73 and 100% in the CCV + CP and CBDV + CP treatment groups, respectively). Compared with controls, percentage mortality increased ($P \leq 0.05$) from 2% to 26 and 34% in the CP and CBDV + CP treatment groups, respectively. Results of this study indicate that the methodology used provides a good model for studying NE.

(*Key words*: necrotic enteritis, *Clostridium perfringens*, chicken, immunosuppression)

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INTRODUCTION

In the poultry industry, *Clostridium perfringens* (CP) is one of the etiologic agents of the disease necrotic enteritis (NE). The disease can be divided into 2 categories, clinical and subclinical NE. Clinical signs of NE include depression, decreased appetite, diarrhea, and severe necrosis of the intestinal tract (Ficken and Wages, 1997). In a survey conducted in 2000, it was estimated that the subclinical form of the disease costs the producer as much as 5 cents per bird, due to decreased performance. Combined with the 1999 broiler meat production estimate, the cost of this disease, including clinical and subclinical infections, was close to 2 billion dollars worldwide (Anonymous, 2000; Van der Sluis, 2000).

Understanding the disease progression of NE has been very difficult due to its complexity and because several predisposing factors such as dietary components, immunosuppression, mechanical irritation of the gut, and sudden gut microflora changes appear to contribute to this syndrome (Smith, 1965; Elwinger et al., 1992; Ficken and Wages, 1997). It has been shown that base components and sudden changes in rations can alter the native microbial population and permit colonization by opportunistic bacteria such as CP (Smith, 1965; Nairn and Banford, 1967; Johnson and Pinedo, 1971; Truscott and Al-Sheikhly, 1977; Branton et al., 1987; Riddell and Kong, 1992; Apajalahti and Bedford, 2000).

Necrotic enteritis is a disease that has been highly correlated with other infectious diseases such as coccidiosis. Coccidiosis causes immunosuppression and severe gastrointestinal damage, which give rise to CP. There have been a number of studies that show a correlation between *Eimeria* and the development of NE (Arakawa and Ohe, 1975; Al-Sheikhly and Al-Saieg, 1979; Frame and Bickford, 1985; Shane et al., 1985). The combination of coccidiosis and necrotic enteritis could have devastating effects on morbidity and mortality in the commercial poultry industry.

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Abbreviation Key: CBDV = commercial bursal disease vaccine; CCV = commercial coccidia vaccine; CP = *Clostridium perfringens*; NE = necrotic enteritis.

Another disease that affects poultry is infectious bursal disease. This virus affects the lymphoid cells and specifically targets the bursa of Fabricius, which is responsible for the development of humoral immunity in chicks. Prolonged immunosuppression has been associated with infectious bursal disease (Lukert and Saif, 1997). Chicks infected with this disease often have secondary infections such as CP and *Escherichia coli* that exacerbate the disease problem. The purpose of the present investigation was to evaluate these diseases on the development of NE following administration of a wheat diet, and the individual and combined effects of a commercial coccidia vaccine[2] (CCV) and a commercial bursal disease vaccine[2] (CBDV).

MATERIALS AND METHODS

Experimental Birds

Ross × Ross straight run broiler chicks were obtained from a local commercial hatchery on day of hatch and were placed on clean pine shaving litter. Birds were reared in 2.4 × 1.2 m pens, with 0.12 m^2 of pen space per bird. Chicks were provided with water and a 55% wheat/corn-based broiler starter diet ad libitum. Using high concentrations of wheat in the diet has been shown to exacerbate the outbreak of necrotic enteritis (Johnson and Pinedo, 1971; Truscott and Al-Sheikhly, 1977; Branton et al. 1987; Riddell and Kong, 1992). The diet met or exceeded National Research Council guidelines for broiler chicks (NRC, 1994).

Experimental Design

Day-of-hatch broilers were assigned to one of the following 8 groups (25 birds/treatment): positive control, CCV, CBDV, CCV + CBDV, and an appropriate nonchallenged negative control for each group in a completely randomized design with 1 pen per treatment and 2 replicate experiments. Birds in the appropriate treatment groups received the commercial vaccines on the 14th day of the experiment. *Clostridium perfringens* was administered via oral gavage to the birds at 10^7 cfu/mL twice daily for 3 consecutive days starting on d 17. On d 25, birds were euthanized and samples were collected. The commercial products (CCV and CBDV) were used as predisposing factors in this disease model. Chicks in the CBDV treatment groups were administered the vaccine at a level 10× the manufacturer's recommended dose via ocular administration (to immunocompromise the chicks). The CCV was administered at a level 24× the manufacturer's recommended dose via oral gavage. Challenge doses, at these concentrations, were chosen based on previous research (data not shown) and have been known to show signs of the disease state with intestinal coccidial lesions, or bursal regression.

Isolation and Administration

Four field isolates of CP (type A) from different geographical locations (1 isolate each from Texas and Virginia, and 2 isolates from Georgia) were cultured separately, then combined and provided to the appropriate treatment groups. The gastrointestinal contents from NE identified birds were shipped overnight to our laboratory where isolation of CP was performed. The gut was sealed with a zip tie below the gizzard and just cranial to the ileo-cecal junction in the lower gut. The sample was placed in a plastic bag and shipped on ice. Upon arrival at our laboratory, the gastrointestinal tract was placed in an anaerobic chamber and approximately 1 g of the gut contents was placed into 10 mL of liquid thioglycollate medium[3] and incubated for 24 h at 37°C. A 10-μL loop was used to streak the bacteria onto *Brucella* blood agar plates,[4] and plates were then incubated (24 h at 37°C). A single colony was removed from the plate and streaked onto a *Brucella* blood agar plate for purity. The bacteria was grown in pure culture and frozen with 20% glycerol at –80°C. For challenge, the isolates were grown in thioglycollate medium for 12 h and the chicks were challenged via oral gavage (3 mL) with 10^7 cfu of CP/mL. Birds were administered CP beginning on d 17 of life, twice daily for 3 d.

Bacterial Culture

To quantitatively measure populations of CP and *E. coli*, a section of the small intestine about 15 to 20 cm in length, cranial to Meckel's diverticulum was removed. The sample was placed in 10 mL of anaerobic thioglycollate, and stomached for 30 s. A 0.5-mL sample of the gut contents was removed and placed into 4.5 mL of neutral PBS. Ten-fold serial dilutions were performed, plated on MacConkey agar,[3] and incubated (24 h at 37°C). Colonies exhibiting typical *E. coli* morphology were counted and recorded. Another 0.5-mL sample was removed from the stomached material, placed into an anaerobic vial containing 4.5 mL of anaerobic thioglycollate, and placed into an anaerobic chamber. The stomached material was incubated for 24 h at 37°C and streaked on blood agar. The following day, the plates were examined for the presence of CP.

Necrotic Enteritis Lesion Scores

To evaluate gross lesions associated with NE, the jejunum and ileum of the small intestine were examined. Lesion scores were recorded using the following criteria (Prescott et al., 1978): 0 = no gross lesions, normal intestinal appearance; 1 = thin-walled or friable, gray appearance; 2 = thin-walled, focal necrosis, gray appearance, small amounts of gas production; 3 = thin-walled, sizable patches of necrosis, gas-filled intestine, small flecks of

[2]Schering Plough Animal Health, Millsboro, DE.
[3]Becton Dickinson Co., Sparks, MD.
[4]Anaerobe Systems, Morgan Hill, CA.

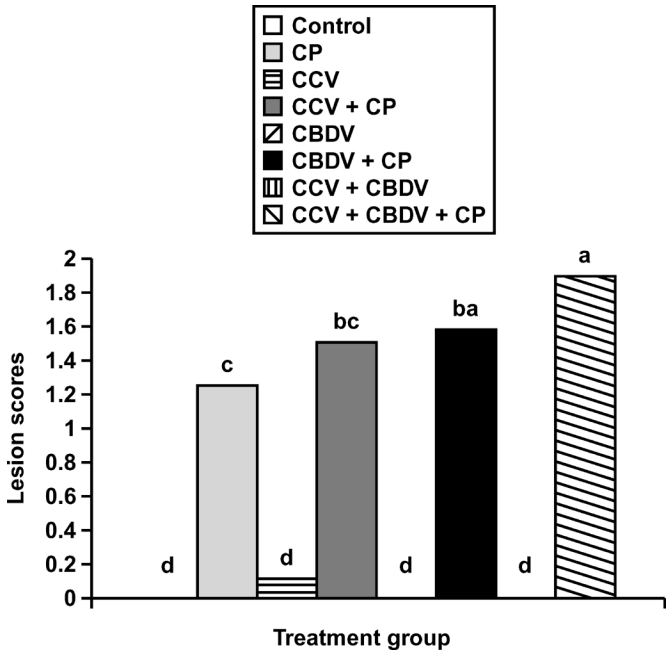

FIGURE 1. Evaluation of lesion scores in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination (CCV + CBDV) in an experimental disease model of necrotic enteritis (2 replicates, n = 50/replicate). a-dValues within treatment groups with different superscripts are significantly different ($P \leq 0.05$).

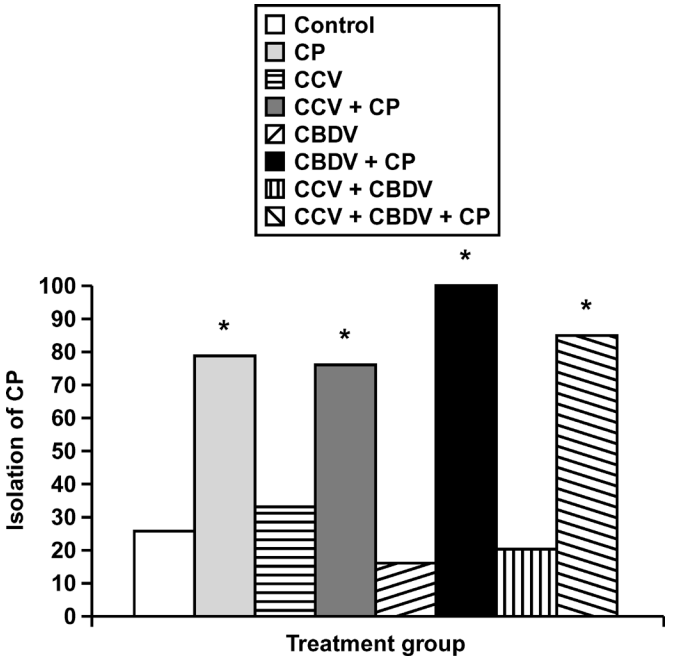

FIGURE 2. Evaluation of *Clostridium perfringens* (CP) isolation frequencies in day-of-hatch broilers fed a wheat diet and challenged with CP in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination (CCV + CBDV) in an experimental disease model of necrotic enteritis (2 replicates, n = 50/replicate). *Treatment groups are significantly different ($P \leq 0.001$).

blood; and 4 = severe extensive necrosis, marked hemorrhage, large amounts of gas in intestine.

Statistical Analysis

Isolation frequencies (+/−) of the recovery of CP from the intestinal cultures were compared using the χ-square test of independence. Mortality and $\log_{10}$ values of *E. coli* were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 1996). Treatment means ($P \leq 0.05$) were separated using Duncan's multiple range test (SAS Institute, 1996). To evaluate the lesion scores in the present investigation, only birds receiving CP were analyzed. The row mean scores were compared using the Cochran-Mantel-Haenszel test. This test showed significant differences ($P \leq 0.05$) and the data were further analyzed using the Kruskal-Wallis test. Nonparametric analysis was performed by ranking the scores, applying the mean to ties, and running an ANOVA on the ranks, allowing the treatment groups to be compared by the mean ranks.

RESULTS AND DISCUSSION

In the present investigation, the development of NE was evaluated with several predisposing factors. All chicks administered CP showed an increase ($P \leq 0.05$) in intestinal lesion scores when compared with nonchallenged controls (Figure 1). The CBDV and the CCV + CBDV treatment groups showed a significant increase in mean lesion scores when compared with the CP-treated chicks. There was no statistical difference between the CP-treated chicks and the CCV-treated birds. This could be due to the challenge dose that was given to the birds or because of the virulence of *Eimeria* strains used in the vaccines. The CCV contains *Eimeria acervulina*, *E. mivati*, and *E. maxima*, which specifically target areas of the digestive tract where typical NE lesions form.

Intestinal coccidia lesions were seen (data not shown), and were consistent with typical coccidial infection. The lesion scores associated with the CCV treatment were low. In the poultry industry, field challenges could be more severe and cause more damage to the GI tract than seen in this experimental model.

The microbial diversity of the gastrointestinal tract dramatically changes when birds are infected with CP. All of the CP-treated chicks had an increase ($P \leq 0.001$) in incidence of CP (Figure 2). Recovery rates were 79, 76, 100, and 85% for CP-, CCV-, CBDV-, and combination-treated groups, respectively. Vaccinated, nonchallenged chicks had low numbers of CP; however, the incidence was not significantly different compared with the control group. The concentration of CP in nonchallenged chicks can be attributed to intrinsic CP bacteria in the gut or to cross contamination between the treatment groups. Interestingly, chicks in the control groups did not exhibit any of the clinical signs associated with the disease upon evaluation of the intestinal tract.

Many populations of bacteria have been shown to coexist with NE. One of the largest populations of bacteria found with NE is *E. coli*. When evaluating populations

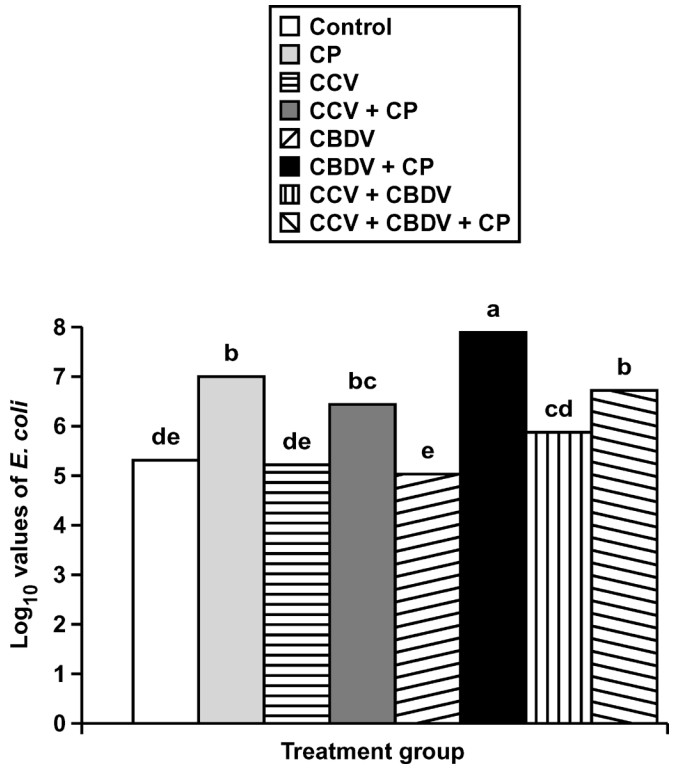

FIGURE 3. Evaluation of *Escherichia coli* in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination (CCV + CBDV) in an experimental disease model of necrotic enteritis (2 replicates, n = 50/replicate). [a–e]Values within treatment groups with different superscripts differ significantly ($P \leq 0.05$).

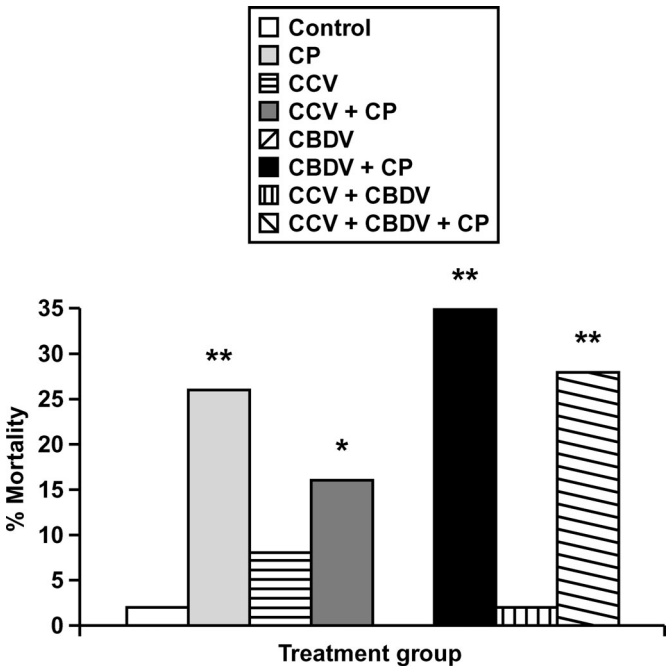

FIGURE 4. Evaluation of mortality in day-of-hatch broilers fed a wheat diet and challenged with *Clostridium perfringens* (CP) in the absence or presence of a commercial coccidia vaccine (CCV), commercial bursal disease vaccine (CBDV), or a combination (CCV + CBDV) in an experimental disease model of necrotic enteritis (2 replicates, n = 50/replicate). *$P \leq 0.025$, **$P \leq 0$ .001.

of intrinsic *E. coli*, increases ($P \leq 0.05$) were seen in all treatment groups that were challenged with CP (Figure 3). The increase in *E. coli* in the control (nonchallenged) CCV + CBDV group can probably be attributed to the immunosuppression caused by both of these products. This data is consistent with experiments performed by Apajalahti and Bedford (2000). Their data showed that chicks with NE that were infected with *Eimeria* had a gut composition that consisted of *E. coli* (52%), CP (14%), Enterococcus spp. (29%), and Proteus vulgaris (5%). These increased levels of *E. coli* are consistent with results of the present investigation and other work performed with NE in our laboratory.

In this disease model, mortality increased ($P \leq 0.025$) in all CP challenge treatment groups (Figure 4). The chicks challenged with CP alone had a mortality rate of 26%, compared with 16, 35, 28, and 2% for the CCV, CBDV, CCV + CBDV, and control treatment groups, respectively.

In the commercial poultry industry, many factors can lead to immunosuppression or infection. To minimize these conditions, broiler flocks are protected by good management practices and therapeutic use of several antibiotics. Coccidiosis has been controlled for many years using ionophores and growth-promoting antibiotics targeting gram-positive bacteria. Although these products have been very effective for controlling coccidiosis, some antibiotics and coccidiostats are losing their effectiveness because of the acquisition of resistance among targeted organisms. It has been well documented that coccidia infections increase the risk of NE (Arakawa and Ohe, 1975; Al-Sheikhly and Al-Saieg, 1979; Frame and Bickford, 1985; Shane et al., 1985). In the present investigation, there were no significant differences between the positive controls and the birds challenged with CCV, except in mortality, in which the treatment group was lower than the positive control. However, when looking at the CBDV-treated birds, there were significant differences between treatment groups and CP positive controls. This data shows that immunosuppression does increase the severity of the disease. In an experimental setting there are many parameters missing (compared with a field outbreak), which could increase the severity of NE. Understanding and evaluating disease progression of NE is important to the poultry industry. The disease model used in this investigation incorporates several of the predisposing factors that commercial poultry operations face daily. This research will provide the industry and researchers with a realistic model to further evaluate this disease condition, and implement management practice and nutritional regimens to minimize the prevalence of NE.

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