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Intestinal bacterial overgrowth includes potential pathogens in the carbohydrate overload models of equine acute laminitis

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ABSTRACT

Carbohydrate overload models of equine acute laminitis are used to study the development of lameness. It is hypothesized that a diet-induced shift in cecal bacterial communities contributes to the development of the pro-inflammatory state that progresses to laminar failure. It is proposed that vasoactive amines, protease activators and endotoxin, all bacterial derived bioactive metabolites, play a role in disease development. Questions regarding the oral bioavailability of many of the bacterial derived bioactive metabolites remain. This study evaluates the possibility that a carbohydrate-induced overgrowth of potentially pathogenic cecal bacteria occurs and that bacterial translocation contributes toward the development of the pro-inflammatory state. Two groups of mixed-breed horses were used, those with laminitis induced by cornstarch ($n = 6$) or oligofructan ($n = 6$) and non-laminitic controls ($n = 8$). Cecal fluid and tissue homogenates of extra-intestinal sites including the laminae were used to enumerate Gram-negative and -positive bacteria. Horses that developed Obel grade2 lameness, revealed a significant overgrowth of potentially pathogenic Gram-positive and Gram-negative intestinal bacteria within the cecal fluid. Although colonization of extra-intestinal sites with potentially pathogenic bacteria was not detected, results of this study indicate that cecal/colonic lymphadenopathy and eosinophilia develop in horses progressing to lameness. It is hypothesized that the pro-inflammatory state in carbohydrate overload models of equine acute laminitis is driven by an immune response to the rapid overgrowth of Gram-positive and Gram-negative cecal bacterial communities in the gut. Further equine research is indicated to study the immunological response, involving the lymphatic system that develops in the model.

1. Introduction

The cecal microbiota changes in the equine carbohydrate overload models of acute laminitis.

Overgrowth of *Lactobacillus* sp. or *Streptococcus* sp. occurs in cecal fluid following infusions of cornstarch (CS) or oligofructan (OF) (Garner et al., 1978; Milinovich et al., 2008). The Gram-negative bacterial communities change as well but precise alterations are less defined. A seven log decrease in cultured enterobacteriaceae was reported following a bolus of CS (Garner et al., 1978). After OF infusion, a time-dependent shift in *Escherichia coli* strains was detected using culture-independent methods

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(Milinovich et al., 2008). In addition to lameness, the horses develop symptoms of systemic inflammation (Belknap et al., 2009). It is not clear how changes in gut microbiota contribute to development of inflammation although bacterial derived factors such as proteases, endotoxin and vasoactive amines are postulated to play a role.

The objective of this study was to explore the possibility that following carbohydrate overload in the horse an overgrowth of potentially pathogenic cecal bacteria occurs and that the bacteria translocate to extra-intestinal organs. Bacterial translocation is a process by which gut-derived bacteria cross the intestinal mucosal membrane and access the lymphatic or circulatory systems (Berg, 1999). The process is involved in the development of mucosal immunity as well as in the pathogenesis of certain food-borne pathogens (Macpherson and Uhr, 2004). If translocation of bacteria occurs and the bacteria localize in tissue, then bacterial metabolites hypothesized to contribute toward the development of acute laminitis could attain physiological active levels in target tissue, the laminae. Culture-dependent and -independent methods were used to enumerate broad groups of potentially pathogenic cecal bacteria and assess the possible contribution of bacterial translocation in carbohydrate overload models of equine acute laminitis.

2. Materials and methods

2.1. Animals

Laminitis is a disease unique to hoofed animals and therefore requires the judicious use of large animals susceptible to the condition. Researchers in the field adhere to the principles of 3Rs of animal experimentation; reduce, replace and refine. In this study, the numbers of animals used was reduced by combining results generated in the course of two separate studies. The control animals in the present study served as controls in an earlier study of chronic laminitis (Onishi et al., 2012). Animals donated and enrolled in the earlier study as control horse were selected from a donor pool after the owner and treating veterinarian determined elective euthanasia was appropriate for non-hoof related disorders where the prognosis was considered poor to grave. Non-hoof related disorders included such conditions as non-healing orthopedic injuries and severe arthritis. Eight mixed breed horses, ranging in age from 8 to 25 years, and consisting of 3 geldings and 5 mares, comprised the control set. The control horses had no abnormalities associated with the hoof and were eating and drinking water normally for their health-status.

Six, mixed breed horses ranging in age from 4 to 5 years and of both sexes were placed on a pelleted ration of Purina Horse Chow. After 7 days, each was given a laminitis-inducing ration of cornstarch wood gruel as described by Garner (Garner et al., 1975). Six mixed breed horses ranging in age from 4 to 9 years and of both sexes were enrolled in a laminitis model induced by oligofructan (inulin) as described by Bailey et al. (2009). Each horse received 1 g/kg body weight of OF top-dressed daily on feed for 3 days prior to induction. Induction involved administering an aqueous solution of 10 g/kg OF by nasogastric intubation on the fourth day. In both induction models, clinical parameters were monitored at 4-h intervals, and included heart rate, respiratory rate, mucous membrane color, capillary refill time, fecal consistency and rectal temperature. Lameness was assessed at 4 h intervals and assigned an Obel lameness score (Menzies-Gow et al., 2010). All animals were euthanized when Obel grade 2 lameness was achieved. Euthanasia protocols used were in compliance with the American Veterinary Medical Association Guidelines on Euthanasia (2007). All procedures were conducted in compliance with regulatory guidelines, and were approved by the Animal Care and Facilities Committee and Rutgers University and the Animal Care and Use Committee at Louisiana State University and at East Tennessee Clinical Research, Inc., Rockwood, TN.

The control horses, as well as those used in the carbohydrate overload models of acute laminitis were de-wormed and vaccinated using accepted veterinary protocols. The control horses for the study were obtained from two facilities in New Jersey. Control horses were maintained on mixed grass pastures common to New Jersey and included Kentucky bluegrass and Orchard grass as the most prominent forages. The animals also had access to timothy hay ad libitum. Experts in the use of the carbohydrate overload models of acute laminitis were located at different facilities. In both cases, test horses were acclimated at research facilities prior to initiation of their respective laminitis induction models. Horses receiving the cornstarch-gruel induction ration were acclimated on Bermuda grass and Bermuda grass hay and Purina Horse Chow for 3 to 4 months prior to enrollment in the study. Horses receiving the oligofructose ration were acclimated for 1 to 7 months on pastures of orchard grass and were provided with timothy-fescue hay. The diets of the control and OF-infused horses were most similar.

2.2. Sample collection

Blood samples were collected prior to euthanasia in sodium polyanethol sulfonate tubes (SPS) (Becton Dickinson, Franklin Lakes, NJ) and cooled on ice. Immediately after euthanasia, the hoof of one forelimb was disarticulated at the fetlock joint and processed for recovery of laminar tissue, as described below. A sterile abdominal field was established and cecal and colonic lymph nodes, liver and spleen were harvested aseptically and placed in sterile containers. A sample of cecal ingesta was collected as the final step in the harvesting process. Sterile tubes containing cecal ingesta were filled to capacity, sealed with parafilm and cooled on ice. Tissues collected from control horses were kept on ice and processed on the day of necropsy. Samples from acute laminitic horses were placed on ice and shipped on the day of necropsy by overnight express delivery to Rutgers University. Culture status was evaluated on the day after necropsy.

2.3. Enumeration of aerobic bacteria in organ homogenates

The laminar tissue was collected and processed as described earlier (Onishi et al., 2012). The laminar tissue