Cattle with increased severity of bovine respiratory disease complex exhibit decreased capacity to protect against histone cytotoxicity[1,2]

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ABSTRACT: Bovine respiratory disease complex (BRDC) is the leading cause of morbidity and mortality in feedlot cattle. Significant inflammation and lesions are often observed in lungs of infected cattle. During acute inflammatory responses, histones contribute to mortality in rodents and humans and serum proteins can protect against histone-induced cytotoxicity. We hypothesized that cattle experiencing chronic or fatal cases of BRDC have reduced ability to protect against cytotoxic effects of histones. Serum samples were collected from 66 bull calves at the time of normal feedlot processing procedures. Animals were retrospectively assigned to groups consisting of calves never treated for BRDC (control [CONT]; n = 10), calves treated with antimicrobials once for BRDC (1T; n = 16), calves treated twice for BRDC (2T; n = 13), calves treated 3 times for BRDC (3T; n = 14), or calves treated 4 times for BRDC (4T; n = 13). Samples were also collected each time animals received antimicrobial treatment; animals within a group were further sorted by calves that recovered and calves that died to test histone cytotoxicity. Bovine kidney cells were cultured in duplicate in 96-well plates and exposed to 0 or 50 μg/mL of total histones for 18 h with 1% serum from each animal. Cell viability was assessed by the addition of resazurin for 6 h followed by fluorescent quantification. Fluorescent values from serum alone were subtracted from values obtained for histone treatment for each animal. Serum from CONT, 1T, and 2T at initial processing all exhibited a similar ($P > 0.10$) response to histone treatment with fluorescent values of –312 ± 557, –1,059 ± 441, and –975 ± 489, respectively. However, 3T and 4T demonstrated an impaired capacity ($P < 0.05$) to protect against histones (–2,778 ± 471 and –3,026 ± 489) at initial processing when compared to the other groups. When sorted by mortality within group, calves that were treated twice and recovered (–847 ± 331) demonstrated a greater ($P < 0.05$) protective capacity than calves that were treated twice and died (–2,264 ± 412), indicating that calves that contract BRDC and ultimately die might have reduced protective capacity against histone cytotoxicity. Results suggest that calves that require multiple treatments for BRDC have reduced ability to protect against cytotoxicity of histones. Understanding the primary mechanism responsible for protecting against histone cytotoxicity could lead to improved identification of animals susceptible to severe cases of BRDC, improved focus and use of available resources, or better treatments for severe cases of BRDC.

Key words: cattle, extracellular histones, histones, respiratory disease, toxicity

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INTRODUCTION

Bovine respiratory disease complex (**BRDC**) is the largest economic problem facing the U.S. cattle industry. Annual economic losses due to death, decreased feed efficiency, and treatment costs associated with BRDC are estimated at US$800 to $900 million (Chirase et al., 2001) and BRDC accounts for approximately 75% of feedlot morbidity and from 50 to 80% of mortality (Edwards, 1996; Chirase et al.,

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2001). Typically, BRDC involves a combination of a viral and bacterial coinfection. Adding to the complexity, not all animals exposed to these pathogens exhibit clinical signs. The disease process can develop sepsis; however, physiological traits for increased susceptibility or increased severity of BRDC remain unclear.

Recently, in humans and mice, extracellular histones have been shown to be involved in death associated with a variety of pathologies including acute sepsis and injury (Xu et al., 2009, 2011). Moreover, histones are lethal when injected into mice (Xu et al., 2011), and serum appears to protect against histone cytotoxicity (Wang et al., 1999). Several serum proteins have demonstrated the ability to bind to histones, and work by Abrams et al. (2013) demonstrated that C-reactive protein (**CRP**) binds to and neutralizes histone toxicity. Therefore, we hypothesize that histone cytotoxicity contributes to the severity of BRDC and that cattle exhibit varying degrees of protective capacity against the cytotoxic effects of histones. The objectives of the current study were to establish an in vitro assay to test protective capacity of serum collected from feedlot cattle against cytotoxic effects of histones and to determine if protective capacity in cattle varies in relation to the degree of severity of naturally acquired BRDC.

MATERIALS AND METHODS

All procedures for the present experiment were approved by the Oklahoma State University Institutional Animal Care and Use Committee (Animal Care and Use Protocol AG-12-11).

Animals

Over a 1-wk period, 355 bulls (BW at arrival = 217 ± 20 kg) were purchased from multiple livestock auctions throughout Oklahoma and transported (average distance = 135 km) to the Willard Sparks Beef Research Center at Oklahoma State University (Stillwater, OK). Upon arrival, calves were visually inspected for abnormalities or illness and subsequently uniquely identified and placed in pens with ad libitum access to hay and water. Calves were allowed to rest 24 to 48 h before initial processing. Initial processing included vaccination against infectious bovine rhinotracheitis virus, bovine viral diarrhea virus types 1 and 2, parainfluenza 3 virus, and bovine respiratory syncytial virus (BRD Shield; Novartis, Greensboro, NC); *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium novyi*, *Clostridium sordellii*, and *Clostridium perfringens* Types C and D (Caliber 7; Boehringer-Ingelheim, St. Joseph, MO); and treatment for the control of internal and external parasites (Ivomec Plus; Merial, Duluth, GA). Animals were surgically castrated by incising the scrotum with a Newberry castrating knife and then emasculation of the cord. Animals were monitored daily for clinical symptoms of BRDC.

The evaluation used criteria based on the depression, appetite, respiration and temperature (**DART**) system (Pharmacia Upjohn Animal Health, Kalamazoo, MI) with some modifications as described by Step et al. (2008). The subjective criteria used for pulling calves consisted of depression, abnormal appetite, and respiratory signs. The evaluators assigned a calf a severity score from 0 to 4 based on the clinical signs and the severity of those signs. A score of 0 was assigned for a clinically normal appearing calf. A score of 1 was assigned for mild clinical signs, 2 for moderate clinical signs, 3 for severe clinical signs, and 4 for a moribund animal. The objective criteria used to determine if antimicrobial treatment was necessary was rectal temperature. All calves assigned a severity score of 1 to 4 were taken to the processing chute for rectal temperature measurement (GL M-500 thermometer; GLA Agricultural Electronics, San Luis Obispo, CA). Any animal that was pulled with a severity score of 1 or 2 and had a rectal temperature of 40°C or greater received an antimicrobial treatment according to label instructions. Any animal with severe clinical signs (severity score = 3 or 4) received an antimicrobial according to label instructions regardless of rectal temperature. For the first antimicrobial treatment, gamithromycin 150 mg/mL (Zactran; Merial) was administered at 1 mL/24.9 kg of BW. After 240 h for calves with a severity score of 1 or 2 and 96 h for calves with a severity score of 3 or 4, florfenicol 300 mg/mL (Nuflor; Intervet/Schering-Plough, Desoto, KS) was administered at the rate of 1 mL/7.56 kg of BW. After 96 h after the florfenicol treatment, calves displaying severity scores of 1 or greater were administered ceftiofur crystalline free acid 200 mg/mL (Excede; Pfizer, New York, NY) at 1 mL/30.2 kg of BW. A 168 h moratorium was observed before administering a fourth antimicrobial treatment (ceftiofur; same dose as described above) as necessary. Postmortem necropsy was also performed on 3 random calves that died on different days within 2 wk after arrival. This included nasal passage swabs and lung tissue sampling. Samples were evaluated by Oklahoma Animal Disease Diagnostic Lab Center for Veterinary Health Sciences at Oklahoma State University (Stillwater, OK).

A subset of 66 bulls was retrospectively assigned to groups consisting of control (**CONT**; n = 10), calves treated with antimicrobials once for BRDC (**1T**; n = 16), calves treated twice for BRDC (**2T**; n

= 13), calves treated 3 times for BRDC (**3T**; n = 14), and calves treated 4 times for BRDC (**4T**; n = 13). For within-group analysis, each group was separated into calves that recovered (**R**) and calves that died (**D**), resulting in 8 different groups (calves that were treated once and recovered [**1TR**; n = 9] or died [**1TD**; n = 7], calves that were treated twice and recovered [**2TR**; n = 6] or died [**2TD**; n = 7], calves that were treated 3 times and recovered [**3TR**; n = 6] or recovered [**3TD**; n = 8], and calves that were treated 4 times and recovered [**4TR**; n = 8] or died [**4TD**; n = 5]).

Serum Collection

Serum was collected at initial processing and each time a calf met the criteria for the standard feedlot definition for treatment of BRDC. A blood sample (9 mL) was collected via jugular venipuncture for subsequent serum analysis (Corvac Serum Separator Tube; Tyco Healthcare Group LP, Mansfield MA). Blood samples were allowed to clot at 4°C overnight and then centrifuged at 1,200 × g for 15 min at 4°C. The separated serum was placed in new tubes and frozen at –80°C until analysis.

Cell Culture

Immortal bovine kidney epithelial cells (Madin-Darby bovine kidney [**MDBK**]) cells were cultured in phenol-free media (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 [**DMEM/F12**; Life Technologies, Carlsbad, CA] supplemented with 5% fetal bovine serum [Invitrogen, Carlsbad, CA] and 1% (vol/vol) 100 IU/mL penicillin and 100 μg/mL streptomycin [Invitrogen]). Cells were incubated at 38.5°C for a minimum of 24 h. Cells were plated into 96-well culture plates at a confluency of 20% and allowed to attach overnight in complete media. After cells attached, media and unattached cells were removed and cells were incubated in serum-free media (DMEM/F12 + antibiotics) in the presence of histones or water control (autoclaved, double deionized water, pH 7.1) and the serum sample.

Histone and Serum Dose Response

To determine the lowest dose of total histones necessary to elicit cytotoxic activity, a histone dose-response was conducted. Initial doses were selected based on previous work demonstrating histone cytotoxicity and serum histone levels in disease (Xu et al., 2009; Abrams et al., 2013). The MDBK cells were treated with a concentration of 0 (control), 25, 50, 100, and 200 μg/mL of total calf thymus histones (Santa Cruz Biotechnology, Dallas, TX) in serum-free media. Cytotoxicity was determined via visual appraisal and resazurin assay as an indicator of cell health (alamarBlue; Life Technologies). The 50 μg/mL of histones resulted in substantial cell death and was selected for subsequent studies (see results). The MDBK cells were then treated with 50 μg/mL of calf thymus histones and serum-free media supplemented with serum from calves not displaying symptoms of BRDC. Serum concentrations of 0, 1, 3, and 5% were tested with serum from 2 separate control animals (cattle with known background that never displayed clinical symptoms of injury or illness and exhibited protective capacity in preliminary experiments). Cell viability was estimated using alamarBlue (Life Technologies) according to manufacturer's recommendations and fluorescence was detected using a Molecular Devices M3 (Molecular Devices, Sunnyvale, CA) plate reader. Cells were incubated with treatment for 18 h and exposed to resazurin for 6 h before reading. Finally, a serum concentration × histone dose response was evaluated using 0.5 and 1% serum and 50 and 100 μg/mL total histones. A serum concentration of 1% and histone concentration of 50 μg/mL was used for subsequent testing. This level of histones was chosen because higher concentrations could have cytotoxic effects despite serum. Likewise, 1% serum was used because the lowest concentration that conferred protection would also be more susceptible to histone cytotoxicity if animal variation in protective capacity existed.

Serum Protective Capacity Assay

Immortal bovine kidney epithelial cells were cultured as described above. Following overnight attachment, cells were incubated with serum-free media + 1% serum from individual calves + vehicle (control) in duplicate wells of a 96-well plate to test for serum effects; duplicate wells were also incubated with phenol serum-free media + 1% serum from individual calves + 50 μg/mL calf thymus histones. Cells were incubated with treatment for 18 h and exposed to resazurin for 6 h before reading. Subsequently, fluorescent values for duplicates were averaged and serum + vehicle was subtracted from serum + histones for each animal. To evaluate potential toxicity of serum alone, each animal's vehicle sample was also compared with fluorescent values from nontreated cells.

Statistics

Fluorescent values from serum samples collected at initial processing and incubated without histones were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) for CONT, 1T, 2T, 3T, and 4T and fluorescent means were estimated with the PDIFF function.

Each animal's normalized fluorescent value estimating histone cytotoxicity (fluorescent value for serum with histones – serum without histones; delta fluorescence) for serum samples collected at initial processing was analyzed using the GLM procedure of SAS for CONT, 1T, 2T, 3T, and 4T. Means were estimated using the PDIFF function and data are presented as least squares means ± most conservative within-group SE.

To analyze histone protective capacity over time in the feedlot, animals that recovered were compared to animals that died within treatment group (1TR vs. 1TD, 2TR vs. 2TD, 3TR vs. 3TD, and 4TR vs. 4TD). Serum samples were collected each time an animal received antibiotic therapy for BRDC and mortality versus recovery was considered treatment. The delta fluorescence was tested against treatment, time, and treatment × time for each group using the MIXED procedure of SAS. Three covariance structures were tested for optimal fit statistics and compound symmetry was chosen. Data are presented as least squares means ± most conservative within-group SE. For all experiments, significance was set at $P < 0.10$.

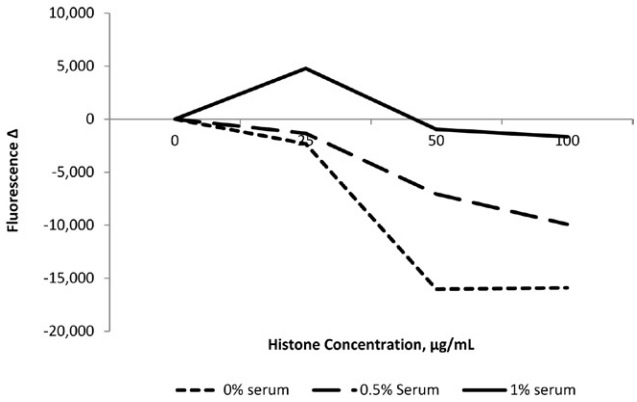

Figure 1. Bovine kidney epithelial cells treated with varying concentrations (0, 25, 50, and 100 µg/mL) of total histones from calf thymus in media containing no serum or serum from control calves (never displayed clinical symptoms of illness; $n = 2$). Control serum was administered at 0, 0.5, and 1% with each histone concentration. Resazurin assay was used as an estimate of cell viability and fluorescent values were obtained 24 h after treatment with histones and serum. The change in fluorescence was calculated with the average of 0 µg/mL of histones as the baseline for each serum concentration. This baseline was subtracted from the corresponding serum concentration at greater histone concentrations to give the change in cell viability due to histone effect. The objective of the experiment was to determine the lowest concentration of serum and lowest dose of histones required to exhibit fluorescent values comparable to control. As in previous experiments (data not shown), results demonstrated that 50 µg/mL of histones was required to induce substantial cell death and 1% serum was required to fully protect against 50 µg/mL total histones.

RESULTS

A marginal decline in MDBK cell viability was demonstrated in cells exposed to 25 µg/mL histones. Increased doses of histones (50 or 100 µg/mL) resulted in a substantial decline in cell viability but there was no difference between 50 or 100 µg/mL (Fig. 1). This devastating response to histones was visually evident as well. Microscopically, 50 µg/mL histones caused significant cell death (Fig. 2). As the lowest dose inducing substantial cell death, 50 µg/mL of histones was used for subsequent serum optimization.

Concentrations of 0, 1, 3, and 5% serum from 2 healthy calves were used in 2 separate assays to evaluate serum protective capacity against 50 µg/mL histones. Data are presented as percent of controls (cells without histones and serum). Without serum, 50 µg/mL histones caused a reduction in cell viability, but addition of the nominal 1% serum restored viability to levels near the histone-free control (Fig. 1). To further refine the serum protective capacity assay, a serum by dose-response experiment was conducted. Healthy calf serum concentrations of 0, 0.5, and 1% were tested against 0, 25, 50, and 100 µg/mL of histones (Fig. 1). The 0% serum concentration showed a response similar to previous optimization steps where 50 and 100 µg/mL histones were extremely toxic to cells. However, no difference was observed between 50 and 100 µg/mL histones. Although 0.5% serum did decrease histone cytotoxicity relative to nonserum samples, this percent was not able to fully protect against histone cytotoxicity. However, 1% serum restored fluorescent values in 50 and 100 µg/mL of histones to near control (histone-free serum) levels. Because 1% serum was the lowest concentration of serum that conferred resistance and 50 µg/mL histones was the lowest dose required to induce cytotoxicity, these were chosen to test the protective capacity of serum in experimental animals.

Serum samples from CONT, 1T, 2T, 3T, and 4T collected at initial processing were evaluated using the resazurin assay. Fluorescent values were obtained in the absence of histone treatment and in the presence of 1% serum for each animal to evaluate toxicity effects of the serum alone and to provide a baseline value for each individual animal. In the absence of histones, there was no difference ($P > 0.20$) in fluorescent values between any of the groups, indicating that there were no toxicity effects of serum alone (Fig. 3A); additionally, there was no difference between serum-treated samples and nontreated cells (data not shown). Cells incubated with 50 µg/mL of histones did not differ in CONT, 1T, and 2T ($P > 0.20$). However, calves that required 3 or more treatments for BRDC exhibited a decreased ($P < 0.05$) capacity to protect against histone cytotoxicity compared with CONT (Fig. 3B) at initial processing. Relative to their own serum sample tested without histones, serum from 3T and 4T had fluorescent values of –2,778 ± 471 and –3,025 ± 489, respectively,

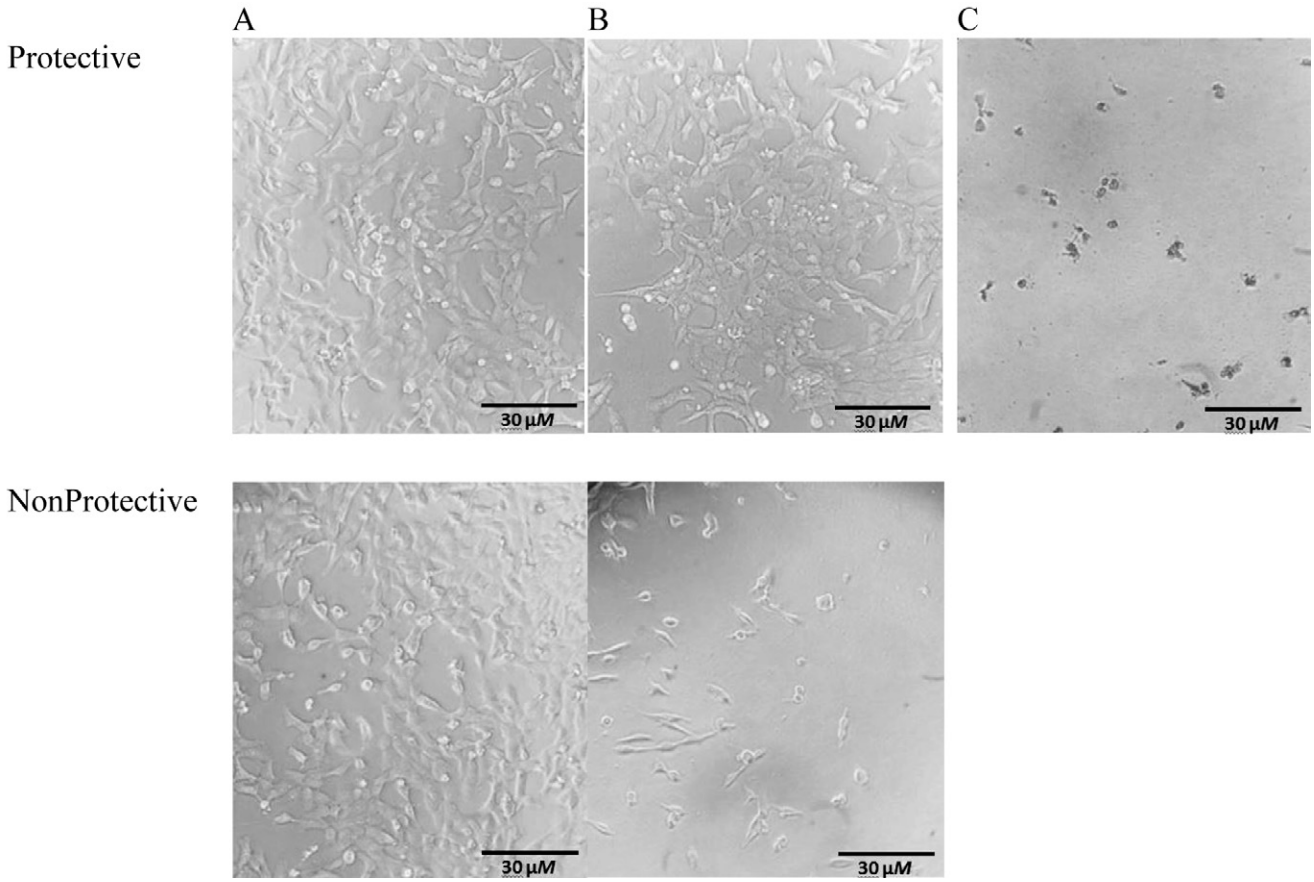

Figure 2. Bovine kidney epithelial cells were treated with 1% serum of representative protective (against histone cytotoxicity) and nonprotective calves and with or without 50 µg/mL total histones. Calves were classified as protective or nonprotective based on previous experiments. Pictures of cells in culture were taken after 24 h treatment with histones, serum, or both. Column A: Cells treated with 1% serum and no histones from a representative protective animal (top) and a representative nonprotective animal (bottom). Column B: Cells treated with 50 µg/mL of histones and 1% serum from a representative protective animal (top) and a representative nonprotective animal (bottom). Column C: Cells treated with 50 µg/mL of histones and no serum.

when incubated with 50 µg/mL histones. Serum samples from CONT were able to protect against histone toxicity and had fluorescent values near their own non-histone baseline (–312 ± 557; Fig. 3B). Calves treated with antimicrobials once for BRDC and 2T displayed protective capacities similar to ($P > 0.10$) CONT and were approximately –1,000 for both groups.

Serum samples were also collected each time an animal received antimicrobial treatment for BRDC, and those that required treatment for BRDC were sorted within group (i.e., 1T, 2T, 3T, or 4T) by calves that recovered and calves that died. To determine if protective capacity against histone cytotoxicity changed between calves that lived and those that died, serum samples within group were analyzed against time, treatment (recovered or died), and treatment × time interactions. For 1T, 3T, or 4T, there was no effect of mortality or time on the protective capacity against histones ($P > 0.10$; Fig. 4A, 4C, and 4D). However, there was a treatment effect for the 2T group. Calves that were treated twice for BRDC and recovered had a greater ($P < 0.05$) overall protective capacity than 2T that ended up dying (Fig. 4B).

DISCUSSION

Bovine respiratory disease complex remains the main cause of morbidity and mortality in feedlot cattle. Despite much research effort, BRDC cases have been relatively constant over the past 30 yr (reviewed by Gifford et al., 2012) and contribute to decreased economic returns due to treatment costs, decreased carcass quality, depressed growth, and death loss. Bovine respiratory disease complex is a multifaceted disease and the etiological agents are typically viral and bacterial or mycoplasmal. The most common viral agents observed are bovine herpesvirus 1, bovine parainfluenza virus 3, bovine viral diarrhea virus 1 and 2, bovine coronavirus, and bovine adenovirus; typical bacterial agents are *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* (reviewed by Griffin et al., 2010). Mycoplasma species can also be pathogens in BRDC outbreaks (reviewed by Griffin et al., 2010). Although pathogens can vary in cases of BRDC, a hallmark of the disease is respiratory tract infections resulting in pneumonia and bronchiolar lesions (Fulton et al., 2009). In the current study, postmortem necropsy of 3 calves identified

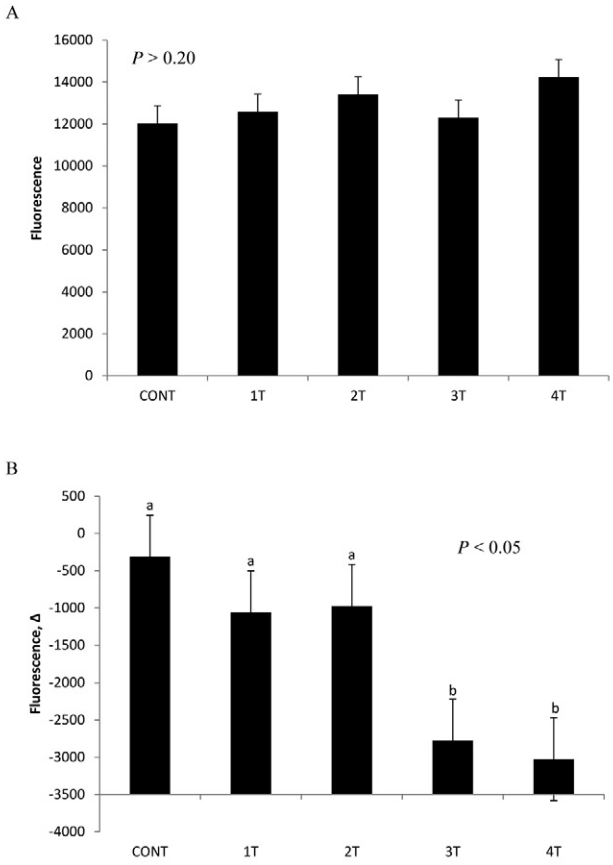

Figure 3. A. Serum samples were collected from 66 bulls at initial processing. Animals were retrospectively assigned to groups consisting of calves that never received antibiotic treatment for bovine respiratory disease complex (BRDC; CONT; $n = 10$), calves treated with antimicrobials once for BRDC (1T; $n = 16$), treated twice for BRDC (2T; $n = 13$), calves treated 3 times for BRDC (3T; $n = 14$), or calves treated 4 times for BRDC (4T; $n = 13$). A) For each animal, 1% serum was incubated alone on bovine kidney epithelial cells (MDBK cells). Resazurin assay was used as an estimate of cell viability and fluorescent values were obtained 24 h after treatment with serum. Data for the 1% serum alone baseline was evaluated using GLM procedure with no significance ($P > 0.20$) between groups. Results show that serum alone collected at initial processing is not cytotoxic. B) For each animal, 1% serum was incubated alone and in combination with 50 µg/mL of histones on MDBK cells. Resazurin assay was used as an estimate of cell viability and fluorescent values were obtained 24 h after treatment with histones. Data from each animal's fluorescent value obtained from serum without histones served as a baseline for that animal. Baseline values were subtracted from respective fluorescent values obtained from incubating MDBK cells with serum + histones. Data are presented as least squares means ± most conservative within-group SE. Columns with different letters indicates significance ($P < 0.05$). Results show that calves that require multiple treatments for BRDC exhibit a decreased capacity to protect against histone cytotoxicity.

several pathogens including *Mannheimia haemolytica*, *Mycoplasma bovis*, bovine viral diarrhea virus, *Bovine coronavirus*, and *Pasteurella multocida* and calves exhibited greater than 85% lung damage. Additionally, the severity of the disease varied both in length (calves requiring only 1 antimicrobial treatment up to 4 treatments) of clinical signs and varied times to death (calves that died after 1 treatment, 2 treatments, 3 treatments, or 4 treatments). The varied severity of disease likely stems from variation of individual resistance (Snowder et al., 2006) and the multiple pathogens involved (Fulton et al., 2009; Kirchhoff et al., 2014). However, for all groups in the current study, the average time to first treatment was 6 ± 1.5 d, indicating that all animals suffered similar exposure on arrival. Ultimately, the resulting experimental group had calves that exhibited varied severity of BRDC ranging from acute to chronic.

Pathologies that induce necrosis (either acute or chronic) or, in some cases, apoptosis cause release of histones into the extracellular spaces (Xu et al., 2009). Histone proteins (H1, H2A, H2B, H3, H4, and H5) help maintain the highly organized structure of DNA in chromatin format within the nucleus of eukaryotic cells. A nucleosome consists of 1 H3/H4 tetramer and 2 H2A/H2B dimers; this histone octomer coils 147 bp of DNA. Together with the linker histones, H1 and H5, nucleosomes form higher order chromatin structures (Felsenfeld and Groudine, 2003). In human patients with acute respiratory distress syndrome and in mice with experimentally induced acute lung injury, extracellular histones are detected in bronchoalveolar lavage fluid (Ward and Grailer, 2014). Likewise, Wen et al. (2013) demonstrated increased circulating histones in a mouse model for acute liver failure and sourced the histones to apoptotic hepatocytes and infiltrating neutrophils. Additionally, there are numerous other diseases and injuries that can cause a rise in circulating histone levels of humans, mice, and baboons (reviewed by Allam et al., 2014), but studies in cattle experiencing BRDC are lacking. Typically, lung damage occurs during episodes of BRDC and other species that suffer acute respiratory damage from disease or injury exhibit a rise in extracellular histones; it is reasonable to speculate that histones are released during BRDC as well. In the current experiment, serum samples were harvested and stored according to normal laboratory procedures. However, nucleosomes in serum are degraded relatively quickly and require stabilization with EDTA to reliably quantify nucleosomes/histones after freezing samples (Holdenrieder et al., 2001). Moreover, samples must be harvested and processed in a similar manner, preferably in under 2 h (Holdenrieder et al., 2001). The samples collected for these data were allowed to clot overnight at 4°C and were not stabilized with EDTA; therefore, it seemed unlikely that circulating histones could be accurately quantified in these samples. If there were increased serum histones in some samples at arrival, it would seem likely those serum samples would be cytotoxic to MDBK cells. There were no detectable differences in cytotoxicity of serum samples from any of the groups at initial processing, indicating that histones or other factors in the samples were not contributing to the observed cytotoxicity.

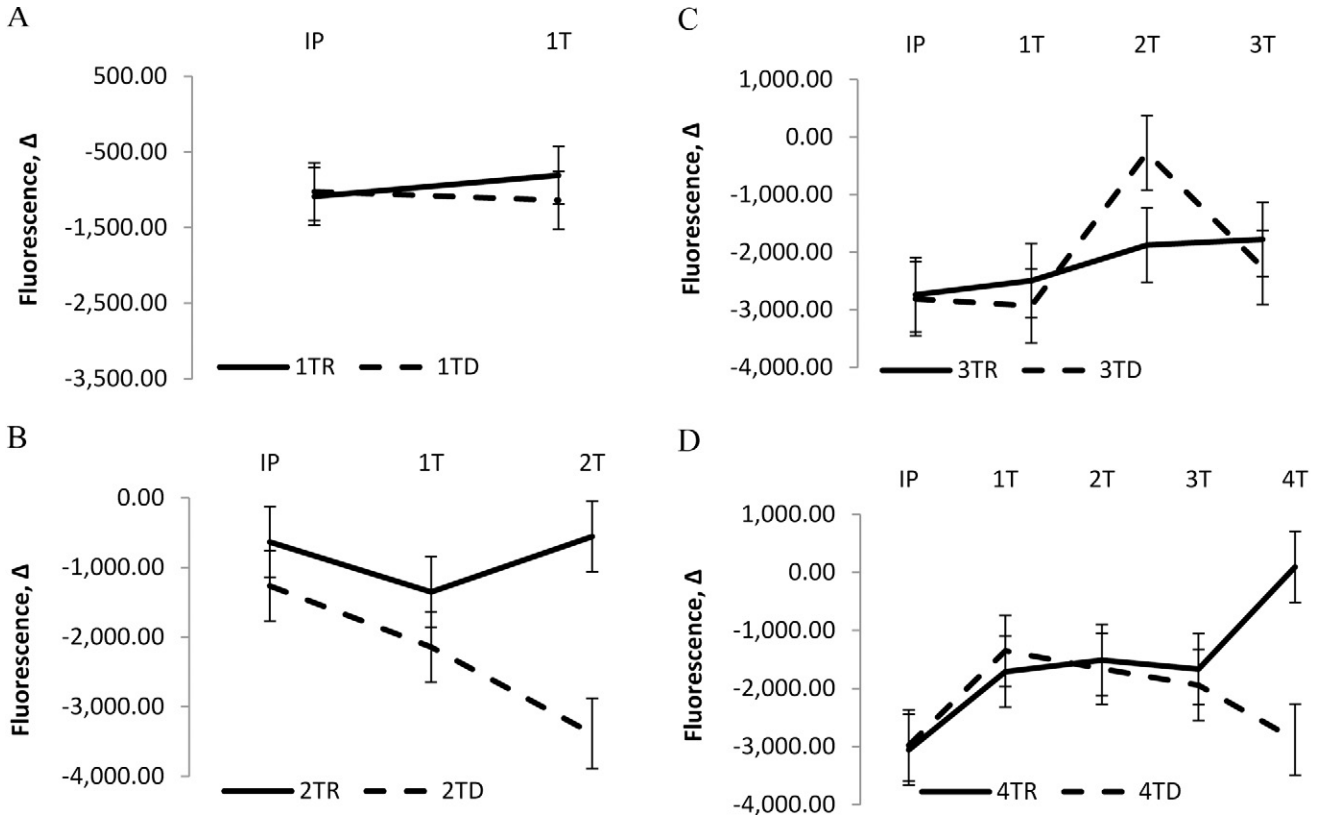

Figure 4. Serum samples were collected from 66 bulls at initial processing (IP) and at each treatment with antimicrobials for bovine respiratory disease complex (BRDC). Calves ($n = 10$) that never received treatment for BRDC are not included in figure. Animals were retrospectively assigned to groups consisting of calves treated once for BRDC ($n = 16$), treated twice for BRDC ($n = 13$), calves that received 3 treatments for BRDC ($n = 14$), or calves that received 4 treatments for BRDC ($n = 13$). Within each group, calves were further assigned as dead after last treatment (D; dashed lines) or recovered after last treatment (R; solid lines). Calves receiving the same total number of treatments were compared across their respective treatments. Samples collected at each treatment are designated as 1T (calves treated with antimicrobials once for BRDC), 2T (calves treated twice for BRDC), 3T (calves treated 3 times for BRDC), and 4T (calves treated 4 times for BRDC). For each animal, 1% serum was incubated alone and in combination with 50 μg/mL of histones on bovine kidney epithelial cells (MDBK cells). Resazurin assay was used as an estimate of cell viability and fluorescent values were obtained 24 h after treatment with histones. Each animal's fluorescent value was calculated with serum without histones serving as a baseline for that animal. Baseline values were subtracted from fluorescent values obtained from incubating MDBK cells with serum + histones. A) Calves that were treated once and died (1TD; $n = 7$) or recovered (1TR; $n = 9$). There was no effect of mortality ($P > 0.10$) on serum protective capacity between calves that recovered and those that died. B) Calves that were treated twice and died (2TD; $n = 7$) or recovered (2TR; $n = 6$). There was an overall effect of mortality on histone protective capacity ($P < 0.05$). Overall, 2TD had a decreased capacity to protect against histone toxicity compared with 2TR. C) Calves that were treated 3 times and died (3TD; $n = 8$) or recovered (3TR; $n = 6$). Overall histone protective capacity was similar for those that died versus those that recovered. D) Calves that were treated 4 times and died (4TD; $n = 5$) or recovered (4TR; $n = 8$). Although initially similar, after 1 treatment 4TD began to show a numerical decline in protective capacity whereas 4TR had a numerical increase in protection against histones after 3 treatments. Nonetheless, there was no overall difference ($P > 0.20$) between calves that died and those that recovered.

The physiological significance of extracellular histones in disease was first demonstrated by Xu et al. (2009), in which an inflammatory challenge led to a rise in extracellular histones and resulted in organ failure and ultimately death during sepsis. Exogenous histones administered intravenously to mice also elicit a hyperinflammatory response leading to cell damage and animal death (Xu et al., 2009). Intratracheal administration of histones greatly induces inflammation leading to epithelial damage, lung consolidation, and death in some cases (Ward and Grailer, 2014). In the absence of serum, we also observed complete cell death in MDBK cells treated with histones. However, histones not only directly induce cell death but can also hyperstimulate the proinflammatory response during a variety of conditions including acute liver failure (Wen et al., 2013). During systemic inflammatory responses from disease or injury, it appears that histones bind toll-like receptors (**TLR**). Using a subfatal dose of histones administered to mice, Xu et al. (2011) found evidence of TLR2 and TLR4 signaling. Furthermore, when injected with exogenous histones, TLR4 knockout mice did not show massive proinflammatory cytokine production. This evidence, in addition to an in vitro model showing direct activation of TLR by exogenous histone treatment, strongly implicates the importance of TLR in histone-induced cytokine production and direct cell or organ damage (Xu et al., 2011).

Increasing evidence supports that there are physiological adaptations to protect against histone toxicity. Aside from identifying extracellular histones as cytotoxic, Xu et al. (2009) noted that activated protein-c

infusion in combination with histones protected mice from the lethal effects of exogenous histones. Treatment with CRP also protected mice from lethal doses of exogenous histones (Abrams et al., 2013). Pemberton et al. (2010) found numerous serum proteins, including the acute phase protein fibrinogen, that bound histones, but the protective capacity of these proteins is undetermined. Results in the present study indicate that increasing the concentration of serum greatly decreased histone toxicity. Collectively, these studies indicate that serum can protect against histone toxicity and that individuals may exhibit varied levels of protection.

In cattle, there is a varied response to the BRDC pathogens among individual animals. Although exposure level and specific pathogen can contribute to varied levels of disease, individual susceptibility may also be important (Snowder et al., 2006). In the current study, calves were physically castrated on arrival; thus, these animals experienced both tissue injury and acute clinical infection. Although not directly measured, it would seem these calves would likely exhibit increased levels of circulating histones. Because histones can exacerbate inflammation, it is interesting to speculate that castration might lead to an increase in extracellular histones. In turn, the extracellular histones might alter the cytokine profile and, along with the stress response, make bulls more susceptible to BRDC.

Nonetheless, we reasoned that, similar to other species, calves would demonstrate varying protective capacity against histone toxicity and those that were least protective would suffer more severe cases of BRDC as indicated by death or duration of illness. Results indicated that serum samples from calves collected at initial processing had varied protective capacities against histone cytotoxicity, and calves that ultimately required 3 or more treatments for BRDC were the least protective. Interestingly, the decreased capacity was observed from serum samples collected at initial processing and before clinical symptoms of BRDC. Certainly, larger studies are necessary, but these data could suggest that histone toxicity protective capacity might be of use to predict which calves will exhibit longer durations of clinical disease and require multiple treatments. Because chronically infected calves experience the most detriment to carcass traits (Holland et al., 2010), perhaps these animals could be managed differently if identified early to minimize economic losses. Ideally, the mechanism or mechanisms for histone protective capacity will be found, leading to new treatments or genetic screening of susceptible individuals.

At initial processing, there was no difference in protective capacity between calves that recovered and those that died within a group. When groups (1T, 2T, 3T, and 4T) were separated by those that recovered and those that died, there were no treatment, time, or treatment × time interactions for histone protective capacity in the 1T, 3T, and 4T groups. There was a treatment effect for the 2T group, and animals that recovered displayed greater protection against the cytotoxic effects of histones than those that died, but studies with a larger sample size would be necessary to confirm these findings.

We initially predicted that calves that die from BRDC would have decreased capacity to protect against histone toxicity. Although not significant, a trend could be emerging from the current data in which animals that recover from BRDC display increasing protective capacity whereas those that die show decreased protective capacity over time. Results from the current study do not statistically support differences in protective capacity based on mortality, but the concept should be revisited with larger sample sizes or assays that directly measure the protective mechanism or mechanisms against histone cytotoxicity. Additionally, serum histone levels could be quantified in properly handled serum samples. Certainly, other components of BRDC are likely contributors to death and histone cytotoxicity is not the sole contributor to clinical BRDC symptoms. It is also possible that calves that die have a massive rise in extracellular histones overriding any protective capacity; therefore, histone levels as well as protective capacity can be contributors to death in BRDC. Larger studies with appropriately processed serum are necessary to describe histone release during BRDC.

In conclusion, as in other species, cattle serum is able to protect against extracellular histone toxicity. Additionally, individual animals exhibit a varied capacity to protect against histone cytotoxicity, and calves that require multiple treatments for BRDC show an impaired capacity to protect against toxic effects of extracellular histones. Future work is needed to identify components of the serum that confer protection and to quantify extracellular histone levels during BRDC.

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