



Assessment of Plasma Zinc and Total Leukocyte Count in Calves Experimentally Infected with *Mannheimia haemolytica*

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Abstract

Mannheimia haemolytica is the main bacterial pathogen isolated in bovine respiratory disease (BRD), a common disease affecting calves before weaning. Previous research has shown that experimental infection with bovine herpesvirus 1, a respiratory virus, decreases plasma zinc (Zn) levels. However, changes in plasma Zn concentrations in calves experimentally infected with *M. haemolytica* have not been studied thus far. The objective of this study was to evaluate the effect of experimental infection with *M. haemolytica* on plasma Zn concentration in calves. Total leukocyte count and bovine respiratory disease (BRD) clinical score were also evaluated. We conducted a 6-day trial in 14 male Holstein calves randomly assigned to one of two groups, experimental (EG, $n = 8$) and control (CG, $n = 6$). Animals in EG were intrabronchially inoculated with *M. haemolytica* (6.5×10^6 CFU/mL) on day 0 of the trial. Plasma Zn levels were affected by time, treatment, and time by treatment interaction, being lower in EG compared with CG on days 1, 2, and 3. Differences in total leukocyte count were significant on day 1, observing a tendency on day 3. BRD clinical score differed between groups, being higher in EG throughout the trial. We conclude that experimental *M. haemolytica* infection reduced plasma Zn concentration in clinically ill calves, suggesting that the clinical condition of animals (healthy/ill) should be considered to better interpret plasma Zn values.

Keywords Micronutrient · Cattle · *Mannheimia haemolytica* · Total leukocyte count · Bovine respiratory disease

Introduction

Zinc (Zn) is an essential trace element for normal growth and development of living organisms. It plays a key structural and regulatory role in mammal cell activity [1] and contributes to the normal function of the immune system. Innate and

acquired immunity are affected by Zn deficiency [2], which is responsible for reduced phagocytic and lytic activity of polymorphonuclear cells and monocytes, reduced *natural killer* cell activity, T lymphocyte alterations, thymic atrophy, and lymphopenia [3, 4].

Supplementation with Zn improves the immune response in human beings and bovines [5, 6]. However, Zn overdosing may be harmful. In human beings, high doses of oral Zn (100 mg/day) have been associated with impaired immune function and alterations similar to those caused by Zn deficiency [7, 8]. On the other hand, addition of 75 mg Zn/kg dry matter to a diet covering Zn requirements elicited lower weight gain and feed efficiency in lactating feedlot heifers [9]. Considering the delicate relationship between the essential nature of Zn and its adverse effects, adequate levels of this micronutrient are needed for optimal health. Accordingly, Zn supplementation should be carefully used and limited to individuals with manifest Zn deficiency [10].

Before deciding supplementation, Zn status should be assessed. Plasma Zn concentration is the most widely used indicators of Zn status [11]. However, it is not considered reliable because it may vary due to different factors, without accurately reflecting the current Zn status. In rodents, plasma

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Zn concentration decreases during acute phase response, inflammatory processes, trauma, infection, and stress [12–14]. In bovines under stress and/or disease conditions, changes are scarcely known.

Bovine respiratory disease (BRD) is the most common disease affecting calves before weaning [15] and the major cause of disease and death in feedlot cattle [16]. The disease is the result of a complex interaction among stress, viruses, and bacteria [17]. Among the latter, *M. haemolytica* is the main bacterial pathogen isolated in BRD feedlot cattle [18]. Previous research performed in calves has shown that experimental infection with bovine herpesvirus 1 (BoHV-1) decreases plasma Zn levels, while bovine syncytial virus infection increases them [19–21]. To date, changes in plasma Zn concentrations in calves experimentally infected with *M. haemolytica* have not been studied.

Based on the hypothesis that *M. haemolytica* infection reduces plasma Zn concentration, thereby affecting its use as a measure of Zn status, the aim of this study was to assess the effect of experimental *M. haemolytica* infection on plasma Zn concentration in calves.

Materials and Methods

All the experimental procedures performed in the current trial were approved by the Committee for the Care and Use of Laboratory Animals (CICUAL, for its Spanish acronym), School of Veterinary Sciences, La Plata National University, Argentina, under Protocol N° 55-2-18P.

Animals

Male Holstein calves ($n = 14$; 3.5 ± 0.2 months old; 67.7 ± 7.7 kg body weight) from a dairy farm (Manantiales, Buenos Aires Province, Argentina; $35^\circ 44' 31.5''S$ $58^\circ 06' 11.7''W$) were used. The animals were housed and kept in individual tie-stalls in the Large Animals Teaching Hospital facilities of the National University of La Plata School of Veterinary Sciences ($34^\circ 91' 20''S$, $57^\circ 93' 75''W$). Calves were fed with alfalfa hay ad libitum and a specially formulated commercial pellet (1.8 kg/day/animal) according to the age and weight of the animals. The pellet provided 15,000 IU vitamin A, 5000 IU vitamin D, 50 mg vitamin E, 90 mg iron, 12.5 mg copper, 60 mg manganese, 90 mg Zn, 0.3 mg selenium, 1.0 mg iodine, and 0.5 mg cobalt per kilogram. At the beginning of the trial, calves were homogeneous with respect to body weight, age, and feed intake. They had good health, and clinical, hematological, and plasma Zn parameters are within the normal range for age and species. After an adaptation period of 12 days to both facilities and food intake (Table 1), they were included in the study.

Table 1 Hematological parameters and plasma Zn levels of the studied animals

Animal ID	Plasma Zn concentration $\mu\text{g/dL}^a$	Leukocytes $10^3/\mu\text{L}^b$
1	93	8.3
2	118	6.7
3	108	10.6
4	111	7.9
5	112	9.8
6	92	10.7
7	91	9.5
9	88	5.5
10	113	8.9
11	109	10.8
12	89	9.1
13	92	8.6
14	98	6.9
15	72	9.2

^{ab} Values correspond to plasma Zn concentrations before starting the trial and are expressed in micrograms per deciliter. Values correspond to total leukocyte counts before starting the trial and are expressed in thousands per cubic microliter

Experimental Design

Calves were randomly assigned to one of two groups, experimental (EG, $n = 8$) and control (CG, $n = 6$). Animals in EG received an inoculum containing a strain of *M. haemolytica* during the log phase of growth, provided by the Laboratory of Bacteriological Diagnosis of the School of Veterinary Sciences. Inoculum concentration was adjusted to the 0.5 McFarland standard scale (final viable count of 6.5×10^6 colony forming units (CFU) per milliliter (mL) of bacterial inoculum) [18]. Intrabronchial inoculation of both left and right lung was performed at 8:00 a.m. with either 8 ml bacterial inoculum plus 8 ml sterile physiological solution (EG) or physiological solution only (16 mL; CG). The time of inoculation was considered as time 0 of the trial.

Blood Sample Collection

Fasting blood samples were obtained daily by jugular venipuncture at 7:00 a.m. from day 0 (before the experimental infection) to 5 (end of the trial). For plasma Zn assessment, blood was collected in 10-ml plastic tubes previously washed with nonionic detergent and nitric acid and treated with sodium heparin as anticoagulant. For total leukocyte count, plastic EDTA-containing tubes were used. Samples were kept at 4°C until processing.

Sample Processing

For plasma Zn assessment, samples were centrifuged within 60 min after blood extraction at 3000 rpm for 5 min. The plasma thus obtained was deproteinized with 10% (w/v) trichloroacetic acid. The Zn concentration in the supernatant was determined by flame atomic absorption spectrometry using an AAnalyst 200 spectrometer (PerkinElmer, AAS, International Equipment Trading Ltd., Mundelein, USA). Internal (0, 50, 100, and 200 $\mu\text{g}/\text{dL}$ Zn) and quality control standards were used [22].

Total leukocyte count was carried out within 6 h after manual extraction using a Neubauer counting chamber. Briefly, 20 μl of blood was mixed together with 380 μl Türk's solution (final dilution, 1:20) so that erythrocytes were lysed and the leukocyte nucleus was stained. The total number of cells in four squares at the corners of the counting chamber was determined under the low power objective of the microscope (10 \times). The total number of cells per microliter of sample was calculated using the following formula: ((number of white cell count \times 20 \times 10)/4), where 20 is the dilution factor, 10 is the chamber depth, and 4 is the number of square counts [23].

Clinical Assessment

The clinical assessment of calves was performed daily following the criteria of the Wisconsin clinical scoring system for the diagnosis of BRD in dairy cows (www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf) [24]. The system assesses the following five clinical signs: rectal temperature, spontaneous or induced coughing, nasal discharge, ocular discharge, and ear and head position. These signs are assigned values on a 0–3 scale, which are used to determine a total BRD score. Calves in the current trial were categorized as clinically ill if their total score was ≥ 5 .

Humane endpoint criteria were applied to calves severely depressed, unresponsive to stimuli, and unable to stand without assistance. The animals were humanely euthanized following the CICUAL protocol.

Euthanasia and Assessment of Pulmonary Lesions

The euthanasia procedure was performed after complete sedation and deep anesthesia with 10% xylazine-5% ketamine (1 and 4 mL, respectively). Then, the animals were sacrificed using 20 mL concentrated procaine (Eutanásico®, Equi Systems SRL, Buenos Aires, Argentina). Once death was confirmed by absence of corneal reflex by touching the surface of the eyeball and heart beats on auscultation, complete necropsy was carried out. Lungs were removed and percentage lung consolidation was assessed by direct palpation. Final pulmonary lesion was determined with the formula ($0.053 \times$ cranial segment of left cranial lobe

$\%$) + ($0.049 \times$ caudal segment of left cranial lobe $\%$) + ($0.319 \times$ left caudal lobe $\%$) + ($0.043 \times$ accessory lobe $\%$) + ($0.352 \times$ right caudal lobe $\%$) + ($0.061 \times$ right middle lobe $\%$) + ($0.060 \times$ caudal segment of right cranial lobe $\%$) + ($0.063 \times$ cranial segment of right cranial lobe $\%$) [25].

Bacteriological Cultures

Routine bacteriological studies of samples from the injured lung segments of the sacrificed animals were performed at the Laboratory of Bacteriological Diagnosis, School of Veterinary Sciences. Briefly, samples were suspended in 0.7 mL of brain-heart infusion broth. A 100 μL aliquot of the suspension was incubated aerobically at 37 $^{\circ}\text{C}$ for 48 h on tryptic soy agar plates containing 5% defibrinated sheep blood at 37 $^{\circ}\text{C}$. After culture, colonies were selected for further Gram-stained smears. The isolates were subjected to routine biochemical tests, i.e., nitrate reduction, catalase, oxidase, urease, and growth on MacConkey agar.

Statistical Analysis

Plasma Zn variations, total leukocyte count, and BRD score were evaluated using an adjusted linear mixed model with repeated measures over time, implemented in SAS PROC MIXED (9.4). Treatment (infected), time (day), and their interaction were the fixed variables, and animal was the random variable. The SLICE option of SAS was used to detect means when differences were significant. Significance was set at $p < 0.05$ for the main effects, $p < 0.1$ for their interaction, and $p < 0.1$ and < 0.15 for tendency, respectively. Plasma Zn levels, total leukocyte count, and BRD clinical score for different levels of the variable time (day) are presented as least square means \pm the standard error of the means (LSM \pm SEM).

Results

Time, treatment, and time*treatment interaction affected plasma Zn levels ($p < 0.001$, = 0.04, and < 0.001 , respectively), which were lower in EG compared with CG on days 1 (46 vs 108 $\mu\text{g}/\text{dL}$, $p < 0.01$), 2 (71 vs 109 $\mu\text{g}/\text{dL}$, $p = 0.03$), and 3 (69 vs 107 $\mu\text{g}/\text{dL}$, $p = 0.03$), respectively (Fig. 1).

Time (day) elicited a significant effect on total leukocyte count ($p < 0.001$), whereas inoculation with *M. haemolytica* tended to decrease it ($p = 0.07$). Time and treatment showed a significant interaction ($p < 0.001$). Differences in total leukocyte count were significant on day 1 (EG, $16.7 \times 10^3 \mu\text{L}$; CG, $9.8 \times 10^3 \mu\text{L}$; $p < 0.001$), while a tendency was observed on day 3 (EG, $11.5 \times 10^3 \mu\text{L}$; CG, $7.8 \times 10^3 \mu\text{L}$; $p = 0.06$) (Fig. 1).

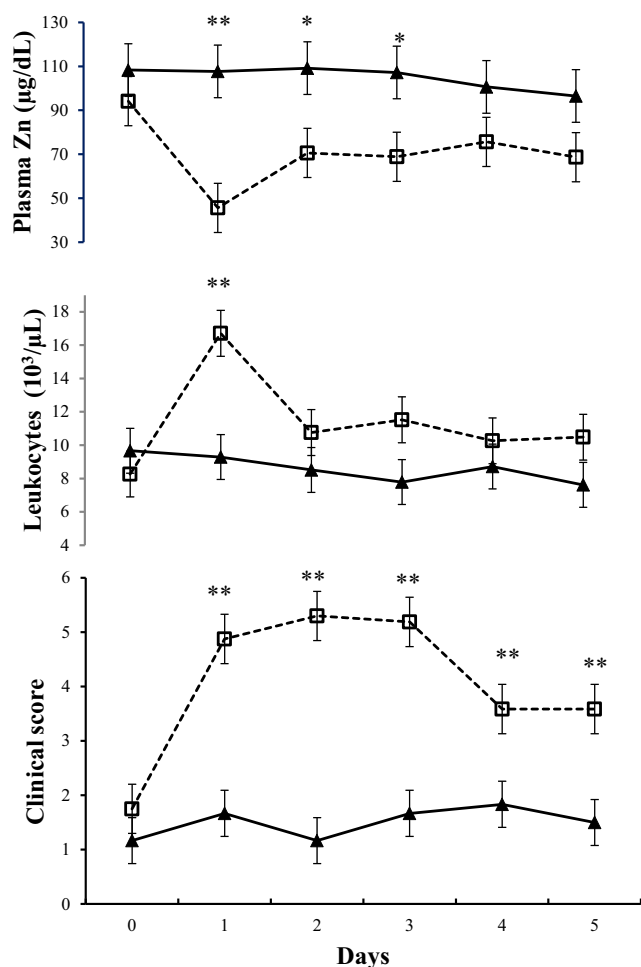


Fig. 1 Plasma zinc concentration, total leukocyte count, and BRD clinical score in calves experimentally infected with *Mannheimia haemolytica*. Day 0: Values recorded before the experimental infection. CG, control group ($n=6$; solid line). EG, experimental group ($n=8$; dotted line) infected with *M. haemolytica* (6.5×10^6 CFU/mL). Three calves from EG were humanely euthanized (one on day 1 of the trial and two on day 2). * $p < 0.05$; ** $p < 0.01$

Time, treatment, and time*treatment interaction affected BRD clinical score ($p < 0.001$), which was higher in EG throughout the trial (Fig. 1).

Fig. 2 Pulmonary consolidation of three calves experimentally infected with *Mannheimia haemolytica*. Pulmonary consolidation (35.46%) was observed in the calf sacrificed on day 1 of the trial (ID 15; 7:30 p.m.). Calves identified as 11 and 12 and sacrificed on day 2 of the trial (5:00 and 7:00 p.m.) presented 40.5 and 42.61% pulmonary consolidation, respectively



Three EG calves with severe depression, unresponsiveness to stimuli, and which did not eat or drink were humanely euthanized. The macroscopic anatomic pathology diagnosis was acute fibrinous bronchopneumonia, without lesions in other organs and systems. At necropsy of the calf sacrificed on day 1 (7:30 p.m.), pulmonary consolidation (35.46%) and plasma Zn concentration of 14 µg/dL were observed. The two calves sacrificed on day 2 of the trial (5:00 and 7:00 p.m.) had 40.5 and 42.61% pulmonary consolidation and 31 and 38 µg/dL plasma Zn level, respectively (Fig. 2).

M. haemolytica was isolated in all bacteriological cultures of samples collected at necropsy of the three animals.

Discussion

According to our hypothesis, experimental infection with *M. haemolytica* decreases plasma Zn concentration in calves. During an infection, the release of interleukin1 β (IL-1 β) and 6 (IL-6) could be involved in plasma Zn reduction [13, 26]. Both cytokines increase Zn transporter ZIP14 gene transcription, mainly in the liver [14]. The localization of ZIP14 at the plasma membrane of hepatocytes facilitates Zn uptake from blood [26]. Additionally, an increased gene transcription of membrane transporter ZIP8 has been described in blood monocytes in response to infection, thereby decreasing plasma Zn content [27]. Such reduction could be due to a redistribution of Zn in the organism as part of nutritional immunity, where pathogenic microorganisms are deprived of essential nutrients needed for survival [28, 29]. Moreover, Zn redistribution increases hepatic Zn levels; the micronutrient participates in liver antioxidant defense system and would reduce oxidative stress damage during infection [30]. The additional provision of Zn to the liver during infection increases liver Zn bioavailability at a time of high Zn requirement due to the increased synthesis of acute phase proteins and cytokines [31].

Studies performed in bovines evaluating other response parameters to bacterial or viral infection have also reported decreased plasma Zn levels. On the one hand, decreased plasma Zn levels 24 h after *Staphylococcus aureus* intramammary infection and increased somatic cell elimination in milk [32]. On the other, plasma Zn concentrations with concurrent morbidity peak (evidenced by increased rectal temperature, nasal and ocular discharge, anorexy, and depression) were found to be decreased 4 days after intranasal herpes virus 1 inoculation of calves [20].

In our study, after the experimental challenge with *M. haemolytica*, plasma Zn levels in EG calves were below 80 µg/dL, which is the lower limit of the marginal range described in the literature [33]. The lowest Zn levels were recorded in euthanized animals, suggesting their association with the most severe lesions caused by *M. haemolytica*. This finding was reinforced by the fact that the lowest Zn values were recorded on the day with the highest total leukocyte count and BRD clinical score. *Mannheimia haemolytica* produces a localized lesion in the lungs; thus, the decrease in plasma Zn could be attributed to a systemic response to infection, which would involve proteins of the immune response. Interestingly, interleukins such as IL-6 have the capacity of diminishing plasma Zn, also causing leukocytosis characterized by rapid neutrophilia [34]. Although we did not examine plasma IL-6 concentrations in this study, values could be remarkably increased at the time when the animals presented the lowest plasma Zn values and the highest leukocyte count.

Despite plasma Zn concentration is altered during infectious processes, it remains the most widely used indicator to evaluate Zn status in bovines [35]. However, the clinical condition of animals (healthy/ill) should also be considered to avoid diagnostic errors, considering that both Zn deficiency and excess are harmful for animal health.

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Authors' Contributions EMG, JMA and LEF designed the study. RML, NS and GA assisted with data collection. GAM and EMG analyzed the data. LEF and JMA coordinated the experiments and revised the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interests.

Ethical approval All the experimental procedures performed in the current trial were approved by the Committee for the Care and Use of Laboratory Animals (CICUAL, for its Spanish acronym), School of Veterinary Sciences, La Plata National University, Argentina, under Protocol N° 55-2-18P.

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