Original Article
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Stress and pain response after oligofructose induced-lameness in dairy heifers

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Lameness is one of the most painful conditions that affects dairy cattle. This study was conducted to evaluate clinical signs and plasma concentration of several pain and stress biomarkers after oligofructose-induced lameness in dairy heifers. Lameness was induced using an oligofructose overload model in 12 non-pregnant heifers. Clinical parameters and blood samples were obtained at 48 and 24 h and at 6, 12, 24, 36 and 48 h after induction of lameness. Clinical parameters included heart rate, respiratory rate, ruminal frequency and lameness score. Plasma biomarkers included cortisol, haptoglobin, norepinephrine, beta-endorphin and substance P. Differences were observed in all parameters between control and treated heifers. The plasma concentration of biomarkers increased significantly in treated animals starting 6 h after induction of lameness, reaching maximum levels at 24 h for cortisol, 48 h for haptoglobin, 6 h for norepinephrine, 12 h for substance P and at 24 h for beta-endorphin. Overall, our results confirm that lameness associated pain induced using the oligofructose model induced changes in clinical parameters and plasma biomarkers of pain and stress in dairy heifers.

Keywords: biomarkers, clinical parameters, heifers, lameness, pain

Introduction

Animal welfare studies have focused on the distress that animals might experience and have highlighted pain as one of the most important aspects to be addressed [37]. Lameness in dairy cows is a serious welfare issue. Over the last few decades, the importance of lameness in dairy cattle has been recognized, and it is becoming one of the most important economic problems afflicting the dairy industry [23]. Lameness is a response to pain caused by trauma, metabolic disorders and infections [15]. Lameness in cattle is a debilitating condition that is often associated with tissue damage, pain and discomfort. This conduction produces a major impact on the productivity of dairy cattle, reducing milk production, decreasing fertility, and increasing the likelihood of other diseases such as mastitis [14]. The notion that chronic pain is a phenomenon distinct from acute pain is widely accepted as common sense among veterinarians and the public [27]. In contrast, questions regarding whether acute pain causes chronic pain have not been completely resolved, although it is known that some stimuli or continuous nociceptive processes provide the impetus for chronic pain to develop [38].

Blood biomarkers correspond to well defined and specific biochemical substances that have proven useful in the diagnosis and treatment of specific conditions [19]. Pain studies in cattle often use the activity of the hypothalamus-pituitary-adrenal (HPA) axis and the autonomous nervous system (ANS) as indicators of stress or pain. Cortisol has been extensively employed as a stress biomarker in lame cattle [18,32]. Nevertheless, it is well known that cortisol plasma concentration is affected by handling procedures or by the sampling process [21]. Norepinephrine has also been used as a marker of stress or acute pain [16,31], and increases in the serum concentration in lame cattle have previously been reported [18]. Haptoglobin is an acute phase protein synthetized by hepatocytes in response to macrophage mediated secretion of cytokines that increases in serum after inflammatory processes, trauma and stress [7,32]. Substance P has arisen as one of the most specific biomarkers of pain because it regulates the excitability of spinal cord neurons after injury and has been used to assess pain after castration in beef calves [5]. Beta-endorphin is an endogenous opioid peptide synthetized by the hypophysis that is considered to be an important component of the anti-nociceptive system, generating analgesia during

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painful conditions. Although a very important mediator of pain, its concentrations have only been evaluated in pregnant cows [24] and during slaughter [36]. This study was conducted to evaluate clinical signs and plasma concentration of several pain and stress biomarkers after oligofructose-induced lameness in dairy heifers.

Materials and Methods

The Ethics and Bioethics Committee on Animal Use of the Universidad Austral de Chile approved the present study (approval no. 42-2012).

Animals, housing conditions and jugular catheterization

Twelve non-pregnant Holstein Friesian heifers weighing between 250 and 300 kg were used in the present study. Heifers were acquired from the Estación Experimental Agropecuaria Austral Farm. Animals were maintained in the Veterinary Teaching Hospital of the Universidad Austral de Chile, and were housed in specially designed stocks with concrete floors and wood shavings. Heifers were fed 8 kg/day of alfalfa hay while provided with limited access to pasture and *ad libitum* access to fresh water to ensure optimal ruminal function. Each animal had an acclimatization period of at least 10 days to minimize stress during the handling procedures. Approximately 48 h before study commencement, the area over the right jugular vein was clipped and disinfected, after which an endovenous over the wire catheter was placed into the vein under aseptic conditions following infiltration of the puncture site with 1 mL of 25 lidocaine solution. Following placement, the catheter was fixed using nylon sutures and maintained using heparinized 0.9% saline solution.

Experimental groups and oligofructose overload

Heifers were randomly allocated into two groups. The treated group received a dose of 13 g/kg oligofructose (Beneo P95; Orafti Active Food Ingredients, Chile) dissolved in potable water and administered in a volume of 2 L/100 kg BW through oro-ruminal gavage. Three days before study commencement, animals received 5% of the initial oligofructose dose twice a day to gradually adjust the ruminal microflora. The oligofructose model is well known for inducing laminitis (hoof wall inflammation) in ruminants, and it has been previously accepted and used as a laminitis and lameness inducer in heifers, resulting in clinically detected lameness [33,34]. The control group received 6 L of potable water administered through oro-ruminal gavage.

Clinical examination

A complete clinical and a specific musculoskeletal system examination was performed for all animals. Any animal that demonstrated previous signs of discomfort or lameness was excluded from the study and treated accordingly. Clinical parameters were obtained at 48 and 24 h before the induction of the lameness and at 6, 12, 24, 36 and 48 h after induction of lameness. Examination included measurement of the heart rate (HR), respiratory rate (RR) and ruminal frequency (RF). Moreover, a subjective evaluation of locomotion was obtained at the same time points using a specific lameness scoring system (1 to 5) as previously described [29]. To accomplish this, each animal was observed and videotaped while walking in a straight line on a concrete floor (15 m approximately). Lameness scoring was performed by two blinded evaluators (HB and MW).

Blood sampling protocol and biomarker determination

Blood samples from all animals were collected in syringes via the jugular catheter at 48 and 24 h before the induction of the lameness. Additional samples were collected at 6, 12, 24, 36 and 48 h after induction of lameness. Blood samples were transferred to tubes containing EDTA and sodium heparin and refrigerated before processing. Plasma was obtained by centrifugation at 1,500 × g over 15 min, then aliquoted in 1.5 mL tubes (Eppendorf, Germany) and frozen at −70°C until further analysis. All analyses were performed in the Clinical Pathology Laboratory of the Veterinary Clinical Sciences Department and in the Pharmacology and Morphophysiology Institute of the School of Veterinary Sciences of the Austral University of Chile.

Plasma cortisol concentration was measured by solid phase radioimmunoassay (Cortisol Coat-A-Count, DPC) validated for bovine plasma. Intra and inter-assay were 4% and 11%, respectively, while the minimum detectable concentration was 8.13 μg/L. Haptoglobin concentration was determined colorimetrically using a commercial kit validated for bovine plasma (Tridelta PHASE Range Development). Norepinephrine concentration was measured using a commercial sandwich ELISA kit validated for bovine plasma (Cusabio Biotech, China). The detection range fluctuated between 312.5 and 5,000 pg/mL, with a minimum detectable concentration of 156.3 pg/mL. The intra and inter-assay were both 15%. Substance P concentration was measured using a commercial sandwich ELISA kit validated for bovine plasma (Cusabio Biotech). The detection range fluctuated between 0.156 ng/mL and 10 ng/mL, with a minimum detectable concentration of 0.039 ng/mL. The intra and inter-assay were 8% and 10%, respectively. The beta-endorphin concentration was measured using a commercial sandwich ELISA kit validated for bovine plasma (Cusabio Biotech). The detection range fluctuated between 46.88 and 3,000 pg/mL, with a minimum detectable concentration of 11.72 pg/mL. The intra and inter-assay were 8% and 10%, respectively.

Statistical analysis

The mean ± SD were calculated for each variable with the exception of lameness score, for which the median and interquartilic range were calculated. For each variable analyzed in the present study, normal probability plots were generated to assess the normality of the data. A repeated-measures two-way analysis of variance (ANOVA) was performed incorporating treatment, time and interaction as the main effects, and baseline values as covariates. In cases in which significant differences were found, Tukey's HSD post hoc test was performed. Differences were considered significant when p values were < 0.05. Statistical analysis was performed using Graphpad Prism 5.02 (GraphPad Software).

Results

Clinical parameters

Clinical parameters measured were heart rate (HR), respiratory rate (RR), ruminal frequency (RF), and lameness score (LS). Baseline values were obtained from the average of the three measurements previous to lameness induction. Significant differences were observed between control and treated heifers at all sampling times (Table 1). Mean HR values obtained for control animals remained constant during all sampling times ($p > 0.05$). In contrast, HR values for treated heifers increased significantly from baseline to 6, 12, 24 and 48 h after oligofructose administration (Table 1). The mean RR remained constant and within reference values in control heifers. In contrast, the mean RR obtained for treated animals showed a significant increase ($p < 0.05$) after 6 h of oligofructose administration, decreasing ($p < 0.05$) at 12 h. Mean values between groups did not differ, except at 6 h, when significant differences ($p < 0.05$) were observed, with values reaching 45 breaths/min in treated heifers (Table 1). The mean RF showed no differences in the control group. However, a significant decrease ($p < 0.05$) was observed from baseline to 6 h in treated heifers. This decrease remained constant at only 1 cycle/min during all sampling times. Significant differences were also observed between groups at 6, 12, and 24 h after oligofructose administration (Table 1). Control heifers did not show any signs of lameness according to the scoring system used. In contrast, treated heifers showed statistically significant differences ($p < 0.05$) when compared to the control, starting at 6 h and lasting until the end of the evaluation period. Moreover, the median for the lameness score in the treated group increased continuously from 2 (6 h) to 3, 4 and 5 (12, 24 and 48 h, respectively) (Table 1).

Blood biomarkers

For convenience, the three initial values (before oligofructose overload) were used to obtain an average value and set as baseline. Control heifers showed no significant differences in their plasma cortisol concentration during the study period, ranging from 28.6 ± 10.8 to 37.3 ± 11.1 μg/L. In contrast, treated heifers showed a significant increase ($p < 0.05$) in plasma cortisol at 6 h, and their values remained elevated for the entire sampling period, peaking at 24 h with values of 63.5 ± 6.7 μg/L. Moreover, significant differences ($p < 0.001$) were evident between control and treated heifers at 6, 12, 24 and 48 h after induction of oligofructose overload (panel A in Fig. 1). The mean haptoglobin concentration for the control group values fluctuated between 0.18 and 0.20 ng/mL without significant differences. In contrast, treated heifers showed a significant ($p < 0.05$) increase in plasma values, starting at 6 h after lameness induction, and peaking at 48 h, with values reaching 4.05 ± 1.51 ng/mL. This increase led to significant differences ($p < 0.05$) between the treated and control heifers at 6, 12, 24 and 48 h (panel A in Fig. 1). Plasma norepinephrine concentrations did not vary significantly among control heifers ($p > 0.05$), ranging from 680.31 to 689.71 pg/mL. In contrast, the mean plasma norepinephrine concentration of treated heifers increased significantly at 6 h, with values of 967.3 ± 101.5

Table 1. Mean ± SD of heart rate (beats/min), respiratory rate (breaths/min), and ruminal frequency (cycles/min), and median and range of lameness score in control (C) and treated (T) heifers

		Previous	6 h	12 h	24 h	48 h
Heart rate	C	51.1 ± 3.72	51.4 ± 5.25	51.2 ± 6.28	51.2 ± 4.51	51.3 ± 4.76
(beats/min)	T	56.0 ± 3.43^{a}	76.3 ± 2.83^{b,**}	75.3 ± 3.74^{b,**}	85.9 ± 3.07^{c,**}	75.9 ± 3.60^{b,**}
Respiratory rate	C	22.2 ± 3.67	22.2 ± 3.58	22.2 ± 3.45	21.8 ± 3.67	21.6 ± 4.69
(breaths/min)	T	24.4 ± 3.16^{a}	40.9 ± 3.92^{b,**}	25.4 ± 2.71^{a}	26.0 ± 3.09^{a}	25.7 ± 3.43^{a}
Ruminal frequency	C	2.3 ± 0.82	1.9 ± 0.87	2.0 ± 0.81	2.0 ± 0.81	1.9 ± 0.87
(cycles/min)	T	2.6 ± 0.51^{a}	0.7 ± 0.48^{b,*}	0.8 ± 0.78^{b,*}	0.9 ± 0.56^{b,*}	1.1 ± 0.56^{b}
Lameness score	C	1.0 (1.0−1.0)	1.0 (1.0−1.0)	1.0 (1.0−1.0)	1.0 (1.0−1.0)	1.0 (1.0−1.0)
	T	1.0 (1.0−1.0)^{a,**}	2.0 (2.0−2.0)^{b,**}	3.0 (3.0−5.0)^{c,**}	4.0 (3.0−5.0)^{d,**}	4.5 (4.0−5.0)^{e,**}

Different letters indicate statistically significant ($p < 0.05$) differences between sampling times. $^{*}p < 0.05$, $^{**}p < 0.01$ differences between groups.

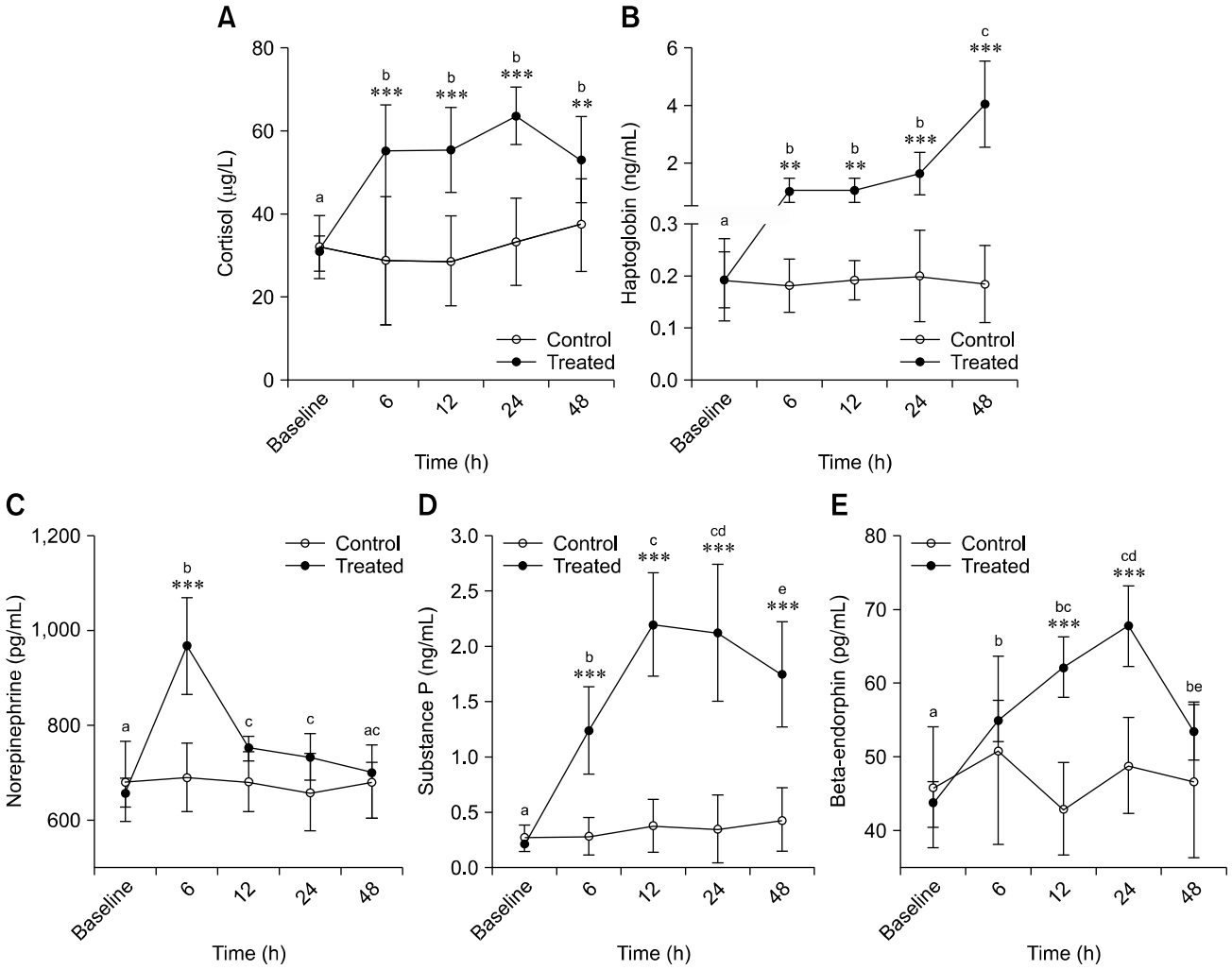

Fig. 1. Mean and standard deviation of plasma biomarkers indicative of lameness-associated pain and stress in dairy heifers after oligofructose overload. (A) Cortisol. (B) Haptoglobin. (C) Norepinephrine. (D) Substance P. (E) Beta-endorphin. $^{**}p < 0.05$ between treatments; $^{***}p < 0.001$ between treatments. Different letters indicate significant differences ($p < 0.05$) between sampling times.

pg/mL being observed. At 12 hours, the values decreased significantly, reaching baseline concentrations (panel C in Fig. 1C). The substance P mean plasma concentration in control heifers ranged from 0.26 ng/mL to 0.42 ng/mL. In contrast, the mean substance P plasma concentration increased significantly ($p < 0.05$) at 6 h after the induction of lameness in the treated group, peaking at 12 h after induction with values of 2.20 ± 0.47 ng/mL, and their values remained elevated at 24 h, then started to decrease 48 h after induction. Significant differences ($p < 0.001$) were observed between control and treated heifers at every time point after baseline (panel D in Fig. 1). No significant differences in the beta-endorphin plasma concentration ($p > 0.05$) were observed among time points in the control group, with values ranging from 42.92 to 50.90 pg/mL. Treated heifers showed a continuous and significant ($p < 0.05$) increase in the plasma concentration of beta-endorphins starting at 6 h after initial oligofructose overload, with values peaking at 67.75 ± 5.48 pg/mL at 24 h after initial treatment. At 48 h, the plasma concentration of beta-endorphins decreased, reaching values similar to those observed at 6 hours. Significant differences ($p < 0.001$) were observed between control and treated heifers at 12 and 24 h (panel E in Fig. 1).

Discussion

The main objective of this study was to evaluate clinical signs and plasma concentrations of several pain and stress biomarkers after oligofructose-induced lameness in dairy heifers. We hypothesized that oligofructose overload and subsequent development of ruminal acidosis would induce lameness in treated heifers. It is well known that metabolic disorders are associated with the onset of inflammatory like laminitis [34].

Increases in heart rate were similar to those previously reported after induction of lameness using the oligofructose model [33]. Although Thoefner *et al*. [33] considered the increase in HR to be related to dehydration and metabolic acidosis, we did not observe signs of dehydration in our animal. We believe that the increases in HR reported here are related to pain, and further associated with increased activity of the sympathetic nervous system (SNS), which was also reflected here by increases in plasma norepinephrine concentration. Several authors have concluded that variations in heart rate and respiratory rate may be valuable indicators of pain [2]. Nonetheless, their values are highly variable and may not be affected by other pathological process of physiological conditions [9]. The reduction of RF is a characteristic sign of acute ruminal acidosis that has been reported in all models of oligofructose overload [6,33]. This decrease in RF further confirms that our oligofructose overload model was successful in inducing ruminal acidosis. All our animals develop lameness as early as 6 h after oligofructose administration. These results differ from those reported previously [33], in which 4 out of 6 animals developed clinically identified lameness. Another important factor that could explain these results is the fact that our lameness scoring analysis was performed from video recordings. According to Whay *et al*. [39], few farmers could positively identify lame cows in their herds [39]. Moreover, scoring analysis disadvantages include being hard to evaluate, low repeatability and the need for expertise to correctly interpret the results [8].

Oligofructose overload induced acute lameness-associated pain. Acute pain induces activation of the hypothalamus-pituitary-adrenal (HPA) axis; therefore, the acute cortisol response can be used to quantify the stress response associated with pain induced by common veterinary practices [21]. Here, cortisol baseline values were higher than those previously reported for sound cows (4.7−7.5 μg/L) [32]. Moreover, we describe increases in plasma cortisol concentration of treated heifers starting at 6 h after oligofructose overload that remained elevated for up to 48 h. This increase indicates that the oligofructose model induces pain and inflammation. Our findings are concordant with those previously reported by Ting *et al*. [35] and Pang *et al*. [25], who described increases in plasma cortisol 24 and 48 h after castration, respectively. Increases as early as 30 min after castration have also been reported [30].

Baseline values for haptoglobin were lower than reference values [32]. The higher haptoglobin plasma concentrations observed here in the treated group are related to the acute insult induced by oligofructose overload. Increases in plasma haptoglobin concentrations have been associated with conditions such as severe sole ulcers, white line abscesses and interdigital dermatitis, which cause a more generalized acute phase reaction [13,32]. Moreover, our results are in agreement with those of Horadagoda *et al*. [11], who concluded that acute phase proteins are useful for differentiating acute from chronic conditions because of their higher sensibility and specificity. This view is supported by Tadich *et al*. [32], who concluded that increases in haptoglobin concentrations were related to lower nociceptive thresholds as lameness score increased, suggesting that secondary lesions may represent persistent inflammatory processes.

Norepinephrine values were similar to those previously reported for ewes and cattle with chronic lameness [17,18]. Plasma catecholamines, which have been used in cattle as a stress biomarker, possess a short half-life [10]. Nevertheless, most studies that describe catecholamines concentrations are associated with short-term painful stimuli such as castration [31] and dehorning [20]. To the best of our knowledge, this is the first study to examine plasma norepinephrine concentrations after oligofructose overload and lameness-associated pain. Here, we describe a significant increase in plasma concentration of norepinephrine 6 h after oligofructose overload. These findings could be associated with stimulation of the SNS in response to pain. Mellor *et al*. [20] previously reported that plasma norepinephrine concentrations peaked at 30 min after dehorning of calves. Moreover, we believe that oligofructose overload induced constant nociceptive stimulation characterized by a marked increase in HR that corresponded with a significant increase of norepinephrine. It is well known that during nociceptive stimulation, norepinephrine modulates and generates pain inhibition [26].

To the best of our knowledge, this is the first report that evaluates changes in plasma concentrations of substance P and beta-endorphin after oligofructose overload and lameness induction. We report significant increases in plasma substance P concentration starting 6 h after oligofructose overload, with the maximum values occurring at 12 h. These results are in agreement with those reported by Coetzee *et al*. [5], who described significant increases in the plasma concentration of substance P in beef calves 45 min after castration. Moreover, significant increases in substance P have been reported after dehorning in calves that did not receive analgesia, with concentrations reaching maximum values 120 h after dehorning [1]. Nonetheless, our results are discordant with the findings reported by Repenning *et al*. [28], who did not find significant differences in the plasma substance P concentration of calves subjected to band castration and their respective controls. Although our results are similar to those of previous studies, several authors have mentioned that sampling time and handling procedures are critical to obtaining reliable substance P concentrations [28,22].

The beta-endorphin concentration for controls were similar to those previously reported for pregnant cows [24]. Our results showed significant differences between control and treated heifers, with values for the treated group peaking 24 h after

induction of lameness by oligofructose overload. Endogenous opioids are relevant to understanding the transition between acute and chronic pain, although the role that beta-endorphins may play as part of the endogenous opioid antinociceptive system is still the subject of debate [3]. According to Chen *et al*. [4], lower analgesia levels associated with dysfunction of the endogenous opioid system are observed in chronic neuropathic pain patients and related to the loss of inhibition induced by μ-opioid receptors in the spinal cord. These opioid receptors are located in the primary afferent fibers and the dorsal horn of the spinal cord, and their primary function is to inhibit the release of excitatory neurotransmitters, specifically substance P [12,40]. These findings suggest that the increases in beta-endorphins observed in our study could be associated with response mechanisms designed to regulate the secretion of Substance P.

We conclude that lameness induced using the oligofructose overload model in heifers was associated with variations in the evaluated clinical parameters. Moreover, the use of specific pain and stress biomarkers was useful in the detection of lameness-associated acute pain characterized by increases in the plasma concentration of cortisol, haptoglobin, norepinephrine, substance P and beta-endorphins. Although the use of these biomarkers could be useful in identifying the mechanisms that mediate the transition between acute and chronic pain in lame cattle, further studies are necessary to confirm our findings.

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Conflict of Interest

There is no conflict of interest.

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