DOI: /10.5513/JCEA01/25.4.4362

Serum lipopolysaccharide concentrations of dairy cows in the pre- and postpartum period and with subclinical laminitis

Sérové koncentrácie lipopolysacharidov dojníc pred a po otelení a so subklinickou laminitídou

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Received: April 12, 2024; Accepted: September 16, 2024

ABSTRACT

This study aimed to compare the lipopolysaccharides (LPS) blood concentrations and metabolic status in the peripartum period and the late lactation phase in dairy cows. In addition, LPS concentrations in animals with subclinical laminitis were compared with those without claw lesions. Blood sampling was carried out on five farms in Central and Eastern Slovakia on Holstein-Friesian dairy cows. Samples were taken from 79 animals. On four farms, clinically healthy animals were sampled and divided into three groups of 5-6 dairy cows each: dairy cows between 10-20 days before calving (Group I), dairy cows 10-20 days after calving (Group II), and dairy cows 4 months after calving (Group III). Within these groups, LPS, BHB, and NEFA concentrations were determined. Five dairy cows with subclinical laminitis and five healthy cows were sampled to determine serum LPS concentrations. LPS were determined using commercial 96-well ELISA kits. BHB and NEFA concentrations (mmol/l) were determined using an automated biochemical analyzer Alizé (Lisabio, Pouilly-en-Aixois, France. Statistical analysis was carried out by a one-way analysis of variance (ANOVA) with the *post hoc* Bonferroni test. One-way ANOVA did not show a significant effect of the lactation period on serum lipopolysaccharides in the study. In addition, the mean LPS concentration in the control dairy cows and cows with subclinical laminitis was 0.048 mg/ml and 0.065 mg/ml, respectively. However, the differences among the groups were not significant. BHB concentrations were within the physiological range in all the groups. In contrast, the cows of the early lactation group showed the highest concentrations of non-esterified fatty acids. In conclusion, elevated NEFA in the post-calving period was not associated with a significant shift in serum LPS concentrations, nor did as well the cows with subclinical laminitis show a strong increase in LPS. The detection of higher LPS concentrations in dairy cows is probably only expected in animals suffering from severe inflammatory diseases, such as ruminitis, enteritis, mastitis, metritis, etc.

Keywords: endotoxins, lipopolysaccharides, laminitis, lipomobilisation, dairy cows

ABSTRAKT

Cieľom tejto štúdie bolo porovnať koncentrácie lipopolysacharidov (LPS) a metabolický stav dojníc v peripartálnom období a v neskoršej fáze laktácie. Okrem toho sa porovnávali koncentrácie LPS dojníc so subklinickou laminitídou a bez ochorení paznechtov. Odbery krvi sa uskutočnili na piatich farmách holštajnského dobytka na strednom a východnom Slovensku. Na štyroch farmách boli odobraté vzorky krvi od 69 klinicky zdravých dojníc, ktoré boli rozdelené do troch skupín po 5 - 6 zvierat: dojnice 10 - 20 dní pred otelením (skupina I), dojnice 10 - 20 dní po otelení (skupina II) a dojnice 4 mesiace po otelení (skupina III). V týchto skupinách sa stanovili koncentrácie LPS, BHB a NEFA. Ďalej boli na stanovenie sérových koncentrácií LPS odobraté vzorky od piatich dojníc so subklinickou laminitídou a piatich so zdravými paznechtami. Koncentrácie LPS boli stanovené pomocou komerčných súprav ELISA. Koncentrácie BHB a NEFA boli merané pomocou automatického biochemického analyzátora Alizé (Lisabio, Pouilly-en-Aixois, Francúzsko. Štatistická analýza sa vykonala jednorozmernou analýzou rozptylu (ANOVA) s *post hoc* Bonferroniho testom. Jednorozmerná ANOVA nepreukázala v štúdii významný vplyv obdobia laktácie na sérové lipopolysacharidy. Priemerná koncentrácia LPS u kontrolných dojníc bola 0,048 mg/ml a u dojníc so subklinickou laminitídou 0,065 mg/ml, bez významného rozdielu medzi skupinami. Koncentrácie BHB boli vo všetkých skupinách vo fyziologickom rozmedzí. Naopak, najvyššie koncentrácie neesterifikovaných mastných kyselín vykazovali dojnice na začiatku laktácie. Záverom možno konštatovať, že zvýšená hodnota NEFA v postpartálnom období nebola spojená s významnou zmenou sérových koncentrácií LPS, a ani dojnice so subklinickou laminitídou nevykazovali signifikantne vyššie LPS. Zistenie vyšších koncentrácií LPS u dojníc sa pravdepodobne očakáva len u zvierat trpiacich závažnými zápalovými ochoreniami, ako sú najmä ruminitída, enteritída a mastitída.

Kľúčové slová: endotoxíny, lipopolysacharidy, laminitída, lipomobilizácia, dojnice

INTRODUCTION

Claw lesions and lameness are common in dairy herds and affect both the welfare and productivity of the animals (Puerto et al., 2021). The group of claw lesions relating to the claw horn is collectively known as claw horn disruptions, some of which are believed to be caused by subclinical laminitis. There have been increased efforts from many laboratories worldwide to improve our understanding of the etiopathogenesis of laminitis and develop new prevention technologies. Interestingly, a growing body of evidence supports the possible role of endotoxins in this highly detrimental disease (Andersen, 2003).

The most commonly discussed subacute ruminal acidosis (SARA) contributes to the development of laminitis through depression of systemic pH and subsequent activation of vasoactive mechanisms that increase blood pressure in the vasculature of the claw. This effect is exacerbated by factors such as histamine, biogenic amines, and endotoxins, and ultimately leads to degradation of microvasculature and subsequent swelling and pain. Vessel damage also limits the transport of nutrients to the epidermal cells and eventually causes degradation of the corium and breakdown of the dermal-epidermis junction (Eades, 2010). Endotoxins are membrane components of gram-negative bacteria (lipopolysaccharides) and gram-positive bacteria (lipoteichoic acids) that elicit a strong immune response when present in the circulation (Draing et al., 2008). The physiological changes (i.e., immunosuppression), the shift to a high grain diet, and the increased exposure to endotoxins [both lipopolysaccharides (LPS) and lipoteichoic acid (LTA)] in many mucosal tissues during the transition period may facilitate the development of these periparturient diseases (Ametaj et al., 2005). Animals experiencing SARA typically exhibit a low-grade inflammation, marked by higher concentrations of circulating LPS, acute-phase proteins (APP), and other inflammatory markers (Fu et al., 2022). Several studies have demonstrated that haptoglobin, apolipoprotein A, and ceruloplasmin are relevant APP in cattle and can be altered in SARA or lame animals (Dong et al., 2015; Fu et al., 2022). Due to not only the structural differences between the ruminal epithelium and that of other tissues but also conflicting results from various studies, it remains unclear whether LPS is, in fact, able to penetrate the rumen in addition to the small intestine and enter the circulation (Plaizier et al., 2012). However, strong evidence exists that during SARA there is a disruption of the ruminal epithelium integrity that would allow the translocation of pathogenic molecules, such as LPS, and increase the risk of inflammation (Minuti et al., 2015). Additionally, some studies have reported evidence of SARA-induced translocation of LPS from the GIT into the circulation which could contribute to systemic inflammation (Emmanuel et al., 2007).

Owing to the increased energy requirements for milk production, the transition from late gestation to the first phase of lactation represents an important metabolic challenge in the current intensive dairy farming. During this period, energy intake does not match the energy required to maintain body condition and milk production, resulting in negative energy balance and increased lipomobilisation (Zachut et al., 2020). Periparturient diseases in dairy cows are very costly for the dairy industry. Among these, ketosis or hyperketonemia predisposes cows to infectious and other metabolic diseases, alters their behaviour, and increases their risk of death or culling (McArt et al., 2015). Recently, it was demonstrated that high blood beta-hydroxybutyrate concentrations were associated with poor reproductive parameters (worse insemination interval and service period) and lower milk production (Ducháček et al., 2023). The beta-hydroxybutyrate (BHB) is a predominant ketone body in the ruminant circulatory system and measuring its concentration is considered the gold standard in the diagnosis of subclinical ketosis, while the determination of non-esterified fatty acid (NEFA) concentrations is used to indicate the degree of lipomobilisation (McArt et al., 2013). Periparturient cows, especially those with clinical ketosis, are in a pro-inflammatory state driven by physiological immune responses and by health events (e.g., heat stress, subacute ruminal acidosis, mastitis, metritis, retained placenta, lameness, pneumonia) that may lead to the entry of bacteria or endotoxins into the bloodstream. A demonstrated increase in adipose tissue lipolysis by lipopolysaccharides compared with unstimulated conditions suggests that inflammation may be a key modulator of lipolysis as it occurs during the early postpartum and clinical ketosis (Chirivi et al., 2023).

The aim of this study was to compare and determine the LPS blood concentrations and metabolic status in the peripartum period and the late lactation phase in dairy cows. In addition, LPS concentrations in animals with subclinical laminitis were compared with those without claw lesions.

MATERIAL AND METHODS

Selection of animals for study

Blood sampling was carried out on five farms in Central and Eastern Slovakia on Holstein-Friesian dairy cows. The farms did not differ in farm hygiene protocols (free stalls with lying boxes) or feeding regimes (TMR twice a day). The lying boxes with concrete surfaces were bedded with straw. The forage component of the TMR that met the animal nutritional requirements included maize and grass silage. The annual milk yield ranged from 9,000 to 11,000 kg. The dairy cows on the farms underwent regular claw trimming twice per year by professional trimmers. Footbaths were not a regular or consistent part of management on these farms. Samples were taken from 79 animals. On four farms, clinically healthy animals were sampled and divided into three groups of 5-6 dairy cows each: dairy cows between 10-20 days before calving (Group I), dairy cows 10-20 days after calving (Group II), and dairy cows 4 months after calving (Group III). Within these groups, LPS, BHB, and NEFA concentrations were determined. On a fifth farm, functional claw trimming of dairy cows was performed, during which subclinical laminitis was diagnosed in five animals. Subclinical laminitis was diagnosed based on typical haemorrhages on the sole or in the white line area and a change in horn colour to yellowish. Blood samples, for the determination of LPS concentration, were taken from animals with subclinical laminitis and from five healthy animals (control group) from the same farm, these dairy cows being at different stages of lactation.

Sample processing

Venous blood was collected from the vena jugularis from 79 dairy cows that were used in this study. Blood collection from 69 clinically healthy dairy cows from the first four farms, was done in the morning (between 8 and 9 am). Venous blood sampling from 5 dairy cows with subclinical laminitis and 5 clinically healthy dairy cows (control group) from the fifth farm, was performed within functional claw trimming and determination of claw health status (subclinical laminitis/healthy claws). Approximately 10 ml of blood was collected in tubes with a gel serum separator without anticoagulant (Meus, Piove di Sacco, Italy). After transporting the blood samples to the laboratory, the serum was separated by centrifugation (3000 g/30 min) and then frozen (-20 C°) until the following day.

LPS values were determined using commercial 96-well ELISA kits. The measurement of lipopolysaccharide concentration by the competitive ELISA method is based on the change in the intensity of the yellow colour in the well. Subsequent spectrophotometric measurement of absorbance (450 nm) was used to calculate LPS concentration (mg/ml).

BHB and NEFA concentrations (mmol/l) were determined using an automated biochemical analyzer Alizé (Lisabio, Pouilly-en-Aixois, France) and the resulting values served as an indicator of the energy metabolism of dairy cows within each group.

Statistical analyses

Statistical analysis was carried out by One-way analysis of variance (ANOVA) with the *post hoc* Bonferroni test. The difference in LPS concentrations between the laminitis and the control group was tested by t-test. Significance was declared at $P < 0.05$.

RESULTS

One-way ANOVA did not show a significant effect of the lactation period on serum lipopolysaccharides in this study (Table 1). However, there was a slight trend towards a certain increase of LPS at the first post-calving sampling point, which correspond with the time of the highest metabolic stress of dairy cows. In addition, the mean LPS concentration in the control dairy cows and cows with subclinical laminitis was 0.048 mg/ml and 0.065 mg/ml, respectively (Figure 1). However, the differences among the groups were not significant.

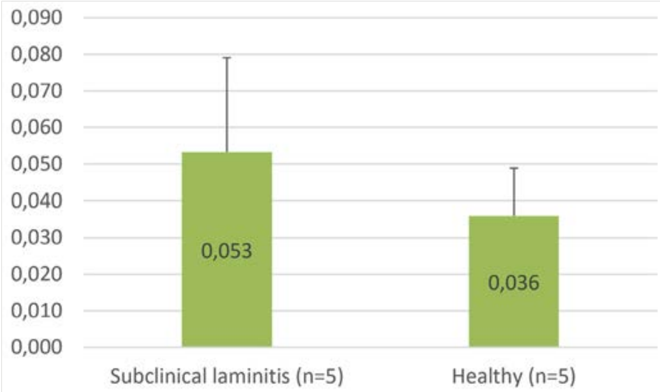

Figure 1. Lipopolysaccharide (LPS) concentrations (mg/ml) in the serum of dairy cows with subclinical laminitis vs. control (healthy) group of dairy cows ($P > 0.05$)

The strongest tendency to metabolic stress was found in the dairy cows in early lactation (group II), however, the measured values of beta-hydroxybutyrate were within the physiological range in all the groups (Table 1). In contrast, the cows of the early lactation group showed the highest concentrations of non-esterified fatty acids ($P < 0.05$), thus demonstrating the highest level of peripheric lipomobilisation.

DISCUSION

The classical theory of laminitis onset was based on the relationship between low rumen pH and laminitis. The rapid drop in rumen pH triggers various systemic reactions (Nocek, 1997) caused by the death of rumen bacteria (Gram-negative) and the release of lipopolysaccharide endotoxins (LPS) from the cell walls of these bacteria into the internal environment of the rumen (Andersen, 2003).

Table 1. Serum concentrations of LPS, BHB, and NEFA in dairy cows at different lactation stage (mean ± SD)

Variables	10 – 20 days before calving (n = 23)	10 – 20 days after calving (n = 23)	4 months after calving (n = 23)	ANOVA
LPS mg/ml	0.011 ± 0.017	0.020 ± 0.029	0.024 ± 0.031	NS
BHB mmol/l	0.41 ± 0.12	0.62 ± 0.54	0.44 ± 0.23	NS
NEFA mmol/l	0.51 ± 0.26^a	1.35 ± 0.81^b	0.71 ± 0.40^c	$P < 0.01$

NS – not significant
a, b, c – values with the different superscripts differ at $P < 0.05$

Subacute ruminal acidosis (SARA) hurts a negative impact on rumen fermentation because low rumen pH leads to reduced fibre digestion, altering the pattern of production of volatile fatty acids, disrupting microbial protein synthesis, leading to altered rumen microflora and less efficient digestion, and ultimately to poor adaptation to a negative energy balance (Kleen and Cannizzo, 2012). The relationship between ruminal acidosis and certain feed characteristics (e.g. high concentration of fast soluble carbohydrates) has been known for decades. More controversial, however, is the relationship between low rumen pH and lameness or laminitis (Passos et al., 2023). Laminitis is often described as one of the symptoms that accompany the onset of SARA (Voulgarakis et al., 2023). Histamine, tyramine, lactic acid, serotonin or LPS are examples of vasoactive substances that are produced in the gastrointestinal tract. Some of these substances can affect vascular perfusion at the level of the bovine claw by their effect on the digital microvasculature, thereby impairing perfusion of the dermis and epidermis tissue (Greenough, 2007). When endotoxins and other vasoactive substances enter the bloodstream, mechanisms of innate immunity that alter the microcirculation can be activated, causing congestion, edema and thrombosis with subsequent hypoxia and necrosis of the chorion, connective tissue, basement membrane and epidermis (Van Amstel, 2009). As a result of these mechanisms, the phalanx distalis sinks and rotates, resulting in compression of the corium in the sole of the claw and the development of lesions in the horn capsule (Randall et al., 2018). Recognition of the initial signs of SARA and subclinical laminitis poses a significant challenge for the dairy industry (Voulgarakis et al., 2024). The most common lesions observed on the claw in subclinical laminitis are haemorrhages on the sole area and yellowish discolouration of the horn (Freitas et al., 2023). The most commonly observed vasoactive agents associated with SARA and laminitis include LPS. Lipopolysaccharides (LPS) are a major component of the cell wall of Gram-negative bacteria. As one of the most important vasoactive substances, LPS play a key role in inflammatory responses. In rumen acidosis, there is a significant death of Gram-negative bacteria and a markedly increased concentration of LPS in the rumen. LPS are absorbed into the bloodstream through the rumen wall until they reach the level of microcirculation in the claw area. LPS cause local inflammatory changes such as activation of cytokines and release of acute phase proteins, thrombocytopenia, leukopenia and subsequently leukocytosis (Shearer, 2013). On the other hand, in the studies of some authors, elevated blood LPS concentrations have not been reported in dairy cows with subacute ruminal acidosis, which does not support the theory of laminitis developing when ruminal pH decreases (Gohzo et al., 2007; Pilachai et al., 2012). The results of this study showed that the LPS concentration was higher in dairy cows with subclinical laminitis, compared to the control group of healthy animals. However, these differences were not significant. These results support the findings of other authors. Guo et al. (2021) reported a significantly higher concentration of LPS in dairy cows with laminitis, compared to healthy cows, while in addition to LPS, a significantly higher concentration of lactic acid was found in animals affected with laminitis. Fu et al. (2022) noted that in addition to elevated blood levels of LPS and lactic acid in animals affected by laminitis, the bacterial composition at the rumen level was also markedly altered. Altered rumen bacterial composition in dairy cows with laminitis was also reported by Guo et al. (2020), who also measured significantly increased concentrations of LPS and lactic acid. These results were confirmed by Zhang et al. (2020), who in their work observed a significant difference in LPS concentration in dairy cows with laminitis compared to healthy dairy cows. In addition, they also compared groups of dairy cows with subclinical and chronic laminitis and found a significantly higher concentration of LPS in the blood in dairy cows with chronic laminitis. Non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) are probably among the most widely used parameters to determine the degree of adaptation of dairy cows during negative energy balance. NEFA concentrations in blood correlate with the degree of fat mobilisation, while BHB concentrations indicate the degree of fat oxidation in the liver, therefore these biomarkers are widely used in the field as indicators of negative energy balance (Mc Art et al., 2013; Ospina et al., 2013) Increases in NEFA concentrations to values greater than 0.3 mmol/l in the antepartum period, and greater than 0.6 mmol/l in the postpartum period, are associated with a higher risk of displacement of abomasum, development of clinical hyperketonemia and laminitis, retained placenta, and metritis (Ospina et al., 2010; Van Saun, 2016). In their study, Mc Art et al. (2013) determined threshold values for NEFA in the prepartum (0.3 - 0.5 mmol/l) and postpartum periods (0.7 - 1.0 mmol/l). Similar results were reported by Mann et al. (2016), who in their study defined dairy cows as suffering from hyperketonaemia during the first two weeks after parturition if the blood β-HB concentration was at ≥ 1.2 mmol/l and the NEFA concentration was at ≥ 1 mmol/l. In agreement with previous studies, the dairy cows included in this study had elevated mean blood NEFA concentrations indicating increased lipomobilisation. Increased mean NEFA concentrations were recorded in all three production groups of dairy cows (0.51 mmol/l, 1.35 mmol/l and 0.71 mmol/l). Based on the measurements recorded in this study, the highest mean concentrations of NEFA and BHB were found in the group of dairy cows 10 to 20 days after parturition, and these results correlate with other studies in which significantly higher NEFA and β-HB values were recorded in dairy cows after parturition, compared to dairy cows in other production groups (Andjelić et al., 2022; Van et al., 2020). Recently, significantly higher contents of milk ketone bodies were found in early and extended lactation than in mid and late lactation (Reindl et al., 2024). Milk has started to be used as a suitable medium for assessing the physiological state because the alterations in the blood are closely mirrored by the composition of milk (Gross and Bruckmaier, 2019). Pechová and Nečasová (2018) reported in their study a significant correlation between elevated BHB concentrations and the prevalence of subacute ruminal acidosis.

CONCLUSION

Based on the results of this study, it is possible to define the period of 10 to 20 days after calving as a critical period, taking into account the metabolic stress of dairy cows during a period associated with an increased risk of negative energy balance. A trend to higher blood LPS concentrations was also measured in dairy cows with subclinical laminitis. However, the differences from healthy dairy cows were not sufficient to conclude that elevated LPS levels directly influence changes in vasculature at the level of the claws. Despite these results, it is important to focus on the effect of LPS concentration in the bloodstream of dairy cows as a possible primary insult responsible for the disruption of the microvasculature of the claws, resulting in the development of pathological changes related to laminitis.

ACKNOWLEDGEMENTS AND FUNDING

This study was supported by the Slovak Research and Development Agency under the contract No. APVV-19-0462.

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