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NMR-based metabolomic evaluation in dairy cows with displaced abomasum

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Abstract: This study is the first to evaluate nuclear magnetic resonance (NMR)-based metabolomics in cows with displaced abomasum (DA), which is an internationally recognized problem in dairy cows. Some biochemical parameters have been used for monitoring DA. However, to date, few data have been available on the blood metabolomic profile of dairy cows. Forty Holstein multiparous cows with DA (30 left, 10 right) and 10 clinically healthy Holstein multiparous cows were the subjects of the study. All the animals had similar dry matter intake. An NMR-based metabolomics approach and hematological and biochemical analyses were performed. Some changes in biochemical parameters were observed between the groups. Among the cows with left displaced abomasum (LDA), 10 were associated with concomitant ketosis. Disease periods between the 2 DA groups were different. The metabolites identified and quantified by NMR analysis were valine, 3 β-hydroxybutyrate (BHB), alanine, glutamine, glutamate, and succinate. The last of these was significantly decreased in cows with right displaced abomasum (RDA). Glutamine, glutamate, and 3 BHB levels were significantly different between DA groups. There was a positive correlation between BHB and valine, glutamine, and glutamate in the LDA group. Overall, this work suggests that the additional information obtained by NMR-based metabolomics evaluation may contribute to assessing the metabolic status of cows with DA.

Key words: Abomasal displacement, dairy cattle, metabolomics, NMR

1. Introduction

Production diseases including displaced abomasum (DA) in dairy cows continue to be a cause of economic loss for the dairy industry and an animal welfare concern (1). Predisposing factors related to DA are breed (e.g., Holstein–Friesian, Simmental–Red-Holstein cross breeds, and Guernsey); genetic background; twin pregnancy; first weeks of lactation; metabolic disorders (ketosis, increased lipomobilization, and insulin resistance); high-concentrate and low-fiber diets; and concomitant diseases such as endometritis, mastitis, and claw disorders (2,3). Recently, with much better management of the chemical and physical properties of dairy cattle rations and their delivery through total mixed rations, the incidence of DA has declined. Nevertheless, this can still be a problem in many situations (4). Research initially focused on treatment and prognosis of the disease. Recently, research has focused more on risk factors, prevention, and prediction of the disease (5).

Blood profiles have frequently been used to assess the nutrient status of cows in the transition period. In addition, body condition score is used in the management of dairy herds. Early blood profiles included packed cell volume and hemoglobin along with glucose, proteins, and minerals. More recently, metabolites such as nonesterified fatty acids (NEFA) and BHB have been added to the profiles to monitor energy balance (6). There are acute-phase responses, oxidative stress, and abomasal tissue damage in DA cases (7–9).

Metabolomics is a branch of 'omics' research that is primarily concerned with the high-throughput identification and quantification of small molecule (<1500 Da) metabolites in the metabolome. Metabolomics is increasingly applied to the identification of biomarkers for disease diagnosis, prognosis, and risk prediction (10). Nuclear magnetic resonance (NMR) spectroscopy is a unique technique that can be applied with high resolution as an initial ex vivo metabolic screening tool (NMR metabolomics) and then translated into in vivo NMR spectroscopy protocols (11).

NMR-based evaluation of metabolomics means simultaneous detection and statistical interpretation of multiple endogenous metabolites. In this pilot study, an NMR-based metabolomics approach was extended to test its ability to identify and quantify physiologically relevant

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metabolites of DA. The aim of this study was to assess the clinical utility of measuring metabolomics by NMR spectroscopy to assess the metabolic status of cows with DA.

2. Materials and methods

2.1. Animals

Forty Holstein, multiparous cows within 1 month of parturition that were presented for treatment at the teaching hospital and diagnosed with DA (30 LDA and 10 RDA) and 10 clinically healthy Holstein cows in early lactation and belonging to the Farm of Faculty of Veterinary Medicine (controls) were the subjects of study. Displacement diagnosis was based on the presence of the characteristic ping on simultaneous auscultation and percussion and exclusion of other causes of left- or right-sided pings. Ultrasonography was helpful in confirming a diagnosis of LDA and RDA. Of cows with LDA, 10 were associated with concomitant ketosis detected by urine chemistry analyzer using urine test strips (Bayer Clinitek 50, Germany) and blood ketone meter using blood ketone test strips (Abbott Optimum Xceed Pro, UK). A Liptak test-needle was placed in the viscus to remove fluid, and pH was measured when needed. All diagnoses were confirmed during a surgical operation. Control animals were also multiparous and within 1 month of lactation and chosen via the same clinical and hematological methods. Control, LDA, and RDA cows had dry matter intake values of 19.4 (7–30), 17.03 (7–30), and 20.4 (7–30), respectively. Disease periods were 8.6 (2–16) days in LDA cows and 3 (1–5) days in RDA cows.

2.2. Hematological analysis

Hematological analyses including complete blood count (blood cell counts, MCV, MCHC, PCV, and Hb) by automated cell counter (MS4e, Melet Schloesing Laboratories, France) and blood gas analysis (pH, HCO_3, and BE) by blood gas analyzer (Gem Premier 3000, Instrumentation Laboratory, USA) were performed in a clinical laboratory.

2.3. Blood chemical profile

Plasma was harvested within 1 h by centrifugation for 15 min at 3000 rpm. Plasma was stored at –80 °C until analysis. It was analyzed for glucose, fructosamine, cholesterol, triglyceride, total protein, Na, K, and ionized Ca; some enzyme (AST, GGT, LDH, and CPK) activities by spectrometry (Autoanalyzer/BT3000 Plus, Italy); and for insulin, NEFA, and BHB by ELISA (Biotek Synergy HT, USA; using East Biopharm commercial kits).

2.4. NMR analysis

Proton (^{1}H) NMR analyses in plasma samples were performed on a 400 MHz spectrometer (Bruker Avance, Germany) using a heteronuclear cryoprobe. The water-soluble extracts were prepared. Briefly, 0.5 mL of ice-cold tissue was mixed with 1 mL of chloroform:methanol (1:1 vol/vol) and centrifuged. The supernatant (organic phase) was collected, and the pellet was resuspended with 0.5 mL of chloroform/methanol and centrifuged. The supernatants were combined, and 0.5 mL of ice-cold water was added to wash out the remaining water-soluble metabolites from the organic phase. After 15 min at –20 °C, the upper (aqueous) phase was removed and added to the remaining pellet (to wash out the remaining water-soluble metabolites from the pellet), 1 mL of water was added, and the sample was centrifuged and freeze-dried overnight. The water-soluble extracts were dissolved in 0.5 mL of D_2O, centrifuged, and transferred into 5-mm NMR tubes. For metabolite identification in plasma water-soluble extracts, a 2-dimensional (2D)-H, C-HSQC (heteronuclear single quantum correlation) was used. For metabolite quantification (proton NMR), a standard water presaturation pulse program (zgpr) was used to suppress water residue signals with a power level of 61 dB (12).

2.5. Statistical analysis

Statistical significance was determined with Kruskal–Wallis test and Mann–Whitney U-test for variables that were not normally distributed. Values of $P < 0.05$ were considered significant. Bonferroni correction was performed to rule out multiple test interference. A 5% significance level was assumed for all variables, and thus 0.05 was divided by the number of tests performed. Therefore, a final $P < 0.017$ for each variable was considered significant. Correlations were examined by Spearman correlation coefficient.

3. Results

Along with spontaneous fluid splashing or pinging sounds, the main observed clinical signs of DA cases in the present study were decreased feed intake, low milk production, reduced frequency of rumen motility and strength of contraction, reduced feces quantity, and slight weakness. All RDA cows had simple, right-sided dilatation, which was confirmed in surgical operations.

There was no alteration in hematological parameters (complete blood counts and blood gas analysis) between groups (Table 1).

Glucose, lactate, and insulin concentrations and some enzyme activities (AST, CK, and SDH) increased significantly, and potassium concentration decreased in both DA groups. BHB and NEFA concentrations increased in LDA cows, and GGT increased in RDA cows alone (Table 2). Among the cows with LDA, 10 were associated with concomitant ketosis. Changes in SDH and cholesterol levels were within the reference ranges.

Metabolites identified and quantified by NMR analysis were valine, 3 β-hydroxybutyrate, alanine, glutamine,

Table 1. Hematological parameters in healthy cows and in cows with LDA and RDL.

	Healthy group (n = 10)	LDA group (n = 30)	RDA group (n = 10)
pH	7.42 ± 0.01	7.45 ± 0.01	7.43 ± 0.03
HCO_3, mmol/L	28.39 ± 1.16	27.73 ± 1.43	28.84 ± 4.02
BE, mmol/L	3.89 ± 1.09	3.39 ± 1.41	3.90 ± 3.77
PCV, %	29.10 ± 1.08	32.23 ± 0.79	31.33 ± 2.36
MCV, fl	44.46 ± 1.50	47.81 ± 0.93	46.56 ± 1.26
MCHC, g/dL	35.80 ± 1.11	33.14 ± 0.60	33.48 ± 0.63
Hb, g/dL	10.12 ± 0.38	10.81 ± 0.28	10.63 ± 0.90
WBC, $10^3/mm^3$	7.60 ± 0.70	8.08 ± 0.88	9.91 ± 190
RBC, $10^6/mm^3$	6.42 ± 0.22	6.96 ± 0.26	6.90 ± 0.63
PLT, $10^3/mm^3$	622.30 ± 79.21	528.67 ± 97.37	543.10 ± 109.05

Table 2. Biochemical parameters in healthy cows and cows with LDA and RDL.

	Healthy group (n = 10)	LDA group (n = 30)	RDA group (n = 10)
BHB, mmol/L	0.50 ± 0.03 [a]	1.55 ± 0.17 [b]	0.64 ± 0.09 [a]
NEFA, µmol/L	79.84 ± 3.22 [a]	111.73 ± 6.83 [b]	93.44 ± 5.31 [ab]
Insulin, µU/mL	7.77 ± 0.48 [a]	11.23 ± 0.53 [b]	10.51 ± 0.76 [b]
Glucose, mg/dL	53.00 ± 4.56 [a]	81.20 ± 3.82 [b]	122.60 ± 13.64 [c]
Fructosamine, µmol/L	210.10 ± 4.53	235.66 ± 6.33	214.00 ± 9.21
Cholesterol, mg/dL	129.20 ± 14.33 [a]	70.21 ± 5.51 [b]	89.60 ± 9.14 [ab]
Triglyceride, mg/dL	21.30 ± 2.18	24.17 ± 1.61	27.90 ± 2.36
T. protein, g/dL	6.87 ± 0.22	6.04 ± 0.18	6.15 ± 0.39
Lactate, mmol/L	0.50 ± 0.09 [a]	2.42 ± 0.37 [b]	4.51 ± 1.34 [b]
SDH, IU/dL	4.21 ± 0.73 [a]	7.40 ± 1.72 [a]	14.60 ± 3.19 [b]
AST, IU/L	80.89 ± 7.50 [a]	149.89 ± 11.66 [b]	163.11 ± 12.93 [b]
GGT, IU/L	33.20 ± 4.79 [a]	49.71 ± 5.85 [ab]	87.90 ± 16.10 [b]
CK, IU/L	198.20 ± 25.87 [a]	664.52 ± 105.49 [b]	638.40 ± 125.68 [b]
Ca, mmol/L	0.88 ± 0.04	0.77 ± 0.02	0.77 ± 0.06
Na, mmol/L	142.50 ± 1.11	140.17 ± 0.85	138.60 ± 0.88
K, mmol/L	3.81 ± 0.08 [a]	3.05 ± 0.10 [b]	2.89 ± 0.19 [b]

Means with different superscripts within a row differ significantly ($P < 0.05$).

glutamate, and succinate (Table 3; Figure). Among these parameters succinate decreased significantly in cows with RDA. Pronounced findings between LDA and RDA groups included significant changes in glutamine, glutamate, and 3 β-hydroxybutyrate. Furthermore, 3 BHB concentrations were positively correlated with valine ($r = 0.313$, $P = 0.043$), glutamine ($r = 0.382$, $P = 0.015$), and glutamate ($r = 0.364$, $P = 0.021$) concentrations in the LDA group.

Table 3. Plasma endogenous metabolites in healthy cows and cows with LDA and RDA.

Metabolites [μmol/mL]	Healthy group (n = 10)	LDA group (n = 30)	RDA group (n = 10)
Valine	7.212 ± 2.861	9.431 ± 0.903	6.135 ± 1.513
3 β-hydroxybutyrate	4.547 ± 1.058 [ab]	8.042 ± 1.256 [b]	2.970 ± 0.784 [a]
Alanine	1.343 ± 0.402	1.149 ± 0.192	0.903 ± 0.238
Glutamine	2.050 ± 0.692 [ab]	2.978 ± 0.411 [a]	1.342 ± 0.416 [b]
Glutamate	1.909 ± 0.659 [ab]	2.348 ± 0.312 [a]	1.056 ± 0.381 [b]
Succinate	1.717 ± 0.365 [a]	0.912 ± 0.096 [ab]	0.592 ± 0.172 [b]

Means with different superscripts within a row differ significantly ($P < 0.05$).

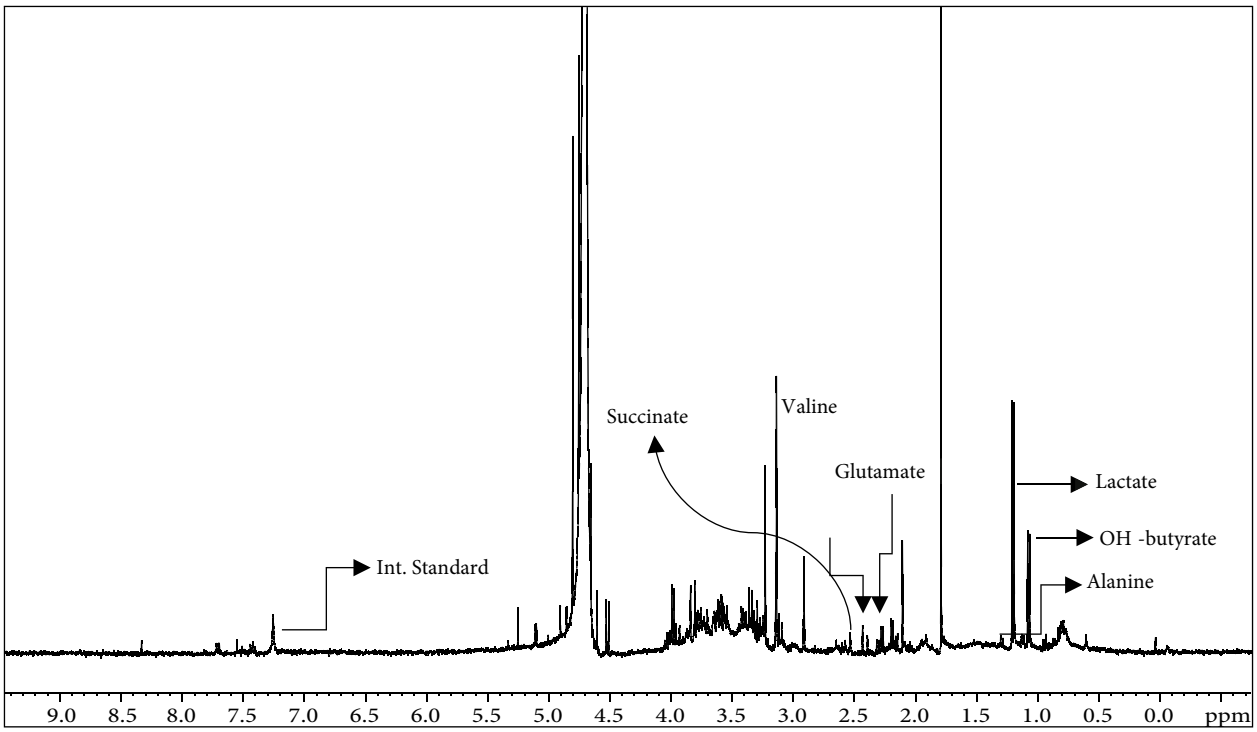

Figure. Representative ^{1}H NMR spectra of plasma water-soluble metabolites.

4. Discussion

Hematological and biochemical analyses were also performed. Generally, a mild metabolic alkalosis with hypochloremia and hypokalemia is common in cows with DA (13). In the present study there was only mild hypokalemia without metabolic alkalosis. Biochemical profiles reflecting energy balance, hepatic function, and inflammatory response are used for the evaluation of peripartum diseases in cows (14). In cows that develop displacement of the abomasum, ketosis may be an important risk factor (3). Cows with DA, in general, have lower feed intake, lower milk production, decreased blood calcium levels, elevated blood ketone body and NEFA concentrations, and high AST activity compared to the matched animals. These preclinical changes may play an important role in the pathogenesis of LDA. Whether there is a causal association between these parameters and LDA, however, is not certain (15). Measurement of NEFA, BHB, and Ca concentrations in the first and second week postpartum may provide useful supplementary information for herd health monitoring (6). Elevated prefresh NEFA concentrations (>0.4 mEq/L) and postfresh BHB concentrations (>1200 μmol/L) are recognized risk factors for ketosis and left-displacement of the abomasum,

respectively (16,17). In general, NEFA is a better predictor of negative downstream outcomes; however, BHB is easy and inexpensive to measure accurately, cow-side, if the appropriate test is chosen (18). NEFA was the parameter that most closely reflected body condition losses, while these losses were not seen in glucose and fructosamine levels (19). In the present study the biochemical parameters, in accordance with the above-mentioned references and our previous work (20,21), reflected a negative energy balance. Although the 2 DA groups of cows had similar dry matter intake, increasing BHB and NEFA concentrations in cows with LDA (of which 10 were ketotic) may be attributed to their longer disease periods.

The spectroscopic techniques applied in metabolomics are often used in a so-called hyphenated mode (e.g., liquid chromatography, mass spectrometry, and NMR); however, NMR-based metabolomics has proven to be particularly apposite for the rapid analysis of complex biological samples. Recently, NMR spectroscopy has become a useful analytical and diagnostic tool in biomedicine (10,11). According to Klein et al. (22), NMR metabolomic analysis of milk in dairy cows reveals the glycerophosphocholine:phosphocholine ratio as a prognostic biomarker for risk of ketosis. In another study (23), where mass-spectrometry–based global metabolomics was used to follow the course of changes in milk and blood plasma during the early stages of lactation, citrate and lactose had the greatest effect on these changes; however, the most significant changes in milk during the first months of lactation were associated with phosphorylated saccharide levels, whereas the most significant changes in blood plasma were associated with levels of polyunsaturated fatty acids containing phosphatidylcholine. In another study (24) in which plasma amino acids were analyzed using an amino acid automatic analyzer, plasma from LDA cattle exhibited significantly higher free fatty acid and BHB; lower glucogenic amino acids such as methionine, alanine, and serine; and a higher ratio of ketogenic amino acids among blood-free amino acids, such as leucine and lysine. In the present study NMR-based evaluation in plasma samples of dairy cows with AD was performed for the first time. The positive correlation between glucogenic amino acids (valine, glutamine, and glutamate) and BHB levels in the LDA group may be attributed to a contribution to glucose production, against ketosis. Succinate is an important biochemical intermediate that occurs in all living creatures. Glutamine is metabolically linked to ammonium detoxification in the body by the hepatocytes. Glutamate is the precursor of glutamine in the glutamine synthetase reaction. Decreased levels of glutamine may reflect increased hepatic dysfunction and/or destruction of hepatocytes (25). Although the results obtained by NMR analysis in the present study may be meaningful and this plot study demonstrated the feasibility of plasma NMR quantitative metabolomics in DA cows, future comprehensive studies including liver metabolomics are needed.

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