Metabolomic analysis of untargeted bovine uterine secretions in dairy cows with endometritis, using ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry

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Abstract

Endometritis is among the most common bovine uterine diseases. It may cause infertility and affect the sustenance and progress of the cattle industry. In this study, a novel metabolomics technique based on ultra-performance liquid chromatography/ quadrupole time-of-flight mass spectrometry was used to compare the uterine secretion metabolomics of healthy cows and those with clinical endometritis, classified based on clinical symptoms. Univariate and multivariate statistical analyses identified significant differences between groups. Compared with healthy uterine secretion samples, coumaric acid, benzoic acid, and equol were downregulated in the clinical endometritis samples. However, L-phenylalanine, glutamine, succinic acid, linoleate, arachidonic acid, and other metabolites were significantly upregulated, revealing significant variations between healthy cows and those with endometritis ($p < 0.05$). This metabolomics approach may provide a more in-depth understanding of the pathobiology of endometritis and a theoretical framework for the diagnosis and treatment of bovine endometritis.

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Running title: Metabolomic markers for endometriosis

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Summary

Endometritis is among the most common bovine uterine diseases. It may cause infertility and affect the sustenance and progress of the cattle industry. In this study, a novel metabolomics technique based on ultra-performance liquid chromatography/ quadrupole time-of-flight mass spectrometry was used to compare the uterine secretion metabolomics of healthy cows and those with clinical endometritis, classified based on clinical symptoms. Univariate and multivariate statistical analyses identified significant differences between groups. Compared with healthy uterine secretion samples, coumaric acid, benzoic acid, and equol were downregulated in the clinical endometritis samples. However, L-phenylalanine, glutamine, succinic acid, linoleate, arachidonic acid, and other metabolites were significantly upregulated, revealing significant variations between healthy cows and those with endometritis ($p < 0.05$). This metabolomics approach may provide a more in-depth understanding of the pathobiology of endometritis and a theoretical framework for the diagnosis and treatment of bovine endometritis.

Keywords : endometritis; uterine secretions; differential metabolites

Introduction

Bacterial infections of the uterus are ubiquitous after parturition in dairy cattle, often causing uterine diseases (Sheldon et al., 2018). According to the severity and clinical manifestations of bacterial infection in the postpartum uterus, uterine diseases can be divided into puerperal metritis, pyometra, and endometritis (Sheldon et al., 2006). Endometritis mainly damages epithelial cells of the endometrium and affects early embryo implantation and other reproductive processes, decreasing the reproductive performance of dairy cows (Sheldon et al., 2002). Among them, clinical endometritis and subclinical endometritis are the most common. Clinical endometritis is characterized by the presence of purulent uterine discharge detectable in the vagina at 21 days or more following parturition, or mucopurulent discharge detectable in the vagina at 26 or more days postpartum (Dubuc et al. 2010). Endometritis is a common and costly disease affecting dairy farms and one of the major reasons for using antibiotics in dairy farming (Sheldon et al., 2010). In China, the incidence rate of endometriosis (20–50%) and infertility (60–90%) in dairy cows can be attributed to endometritis, which severely affects the conception rate and uterine secretion yield of dairy cows (Bao-Qi et al., 2016). These issues represent a bottleneck limiting the development of the cattle industry in China.

Metabolomics is a newly developed "omics" following genomics, transcriptomics, and proteomics. It refers to the analysis of the metabolic response of all small-molecule metabolites (amino acids, glycols, lipids, etc.) in organisms under environmental, temporal, and external stimuli, along with the research methods used to understand the relationship between metabolites, physiological changes, and pathological changes (Bais et al., 2011; Bolten et al., 2007). Briefly, metabolomics focuses on analyzing overall metabolite levels and understanding the metabolic grid, dynamic regulation, and control of metabolic pathways. Modern detection technologies are used to analyze and detect as many metabolites as possible (Tang et al., 2006; Wang et al., 2011). Thus, in this study, ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) coupled with univariate and multivariate statistical analyses were used to compare the uterine secretion metabolomics between healthy cows and those with clinical endometritis. The non-targeted metabolomics method based on UPLC-QTOF-MS has higher resolution and high throughput and can be used for accurate qualitative and quantitative analysis of small- and medium-molecule characteristic compounds from uterine secretions (Boudonck et al., 2009). This study was conducted to identify metabolic signatures and biomarkers of uterine secretions specific to cow endometritis. Such data will further our understanding of endometritis-related metabolic changes and provide information on possible measures for enhancing the diagnosis and treatment of clinical endometritis.

Materials and methods

Ethics statement

Ethical approval for the animal experiments was granted by Gansu Agricultural University (GSAU-AEW-2017-0003). The collection of cow milk samples was permitted by the owner of the farm.

Collection of uterine secretion samples

Cows were selected from a standardized dairy farm at Qianjin Farm (Zhangye, China). Rectal palpation and cervicovaginal mucus observation were conducted to identify healthy animals or cows with endometritis. The opening of the cervix could be felt distinctly during a rectal examination. In repeat breeder dairy cows within three months postpartum, a thick uterine horn, high uterine secretions, and a large amount of yellow or white pus discharged from the vagina were considered as evident symptoms of clinical endometritis (i.e., presence of purulent or mucopurulent vaginal discharge) (Figure 1A). However, the cows with endometritis did not show symptoms of other local or systemic diseases (Sheldon et al., 2006, Williams et al., 2005). In the chemical examination, the uterine secretion was boiled with 4% sodium hydroxide. After cooling, uterine secretion from healthy cows was colorless, whereas a slightly yellow color indicated positivity for endometritis. All cows were fed and managed routinely (Figure 1B) (Li et al., 2010).

A 0.1% potassium permanganate solution was used to disinfect the vulva of the cattle. Three milliliters of

secretion from the uteri of cattle were extracted through a sterile, one-time-use fertilization tube and stored in aliquots at -20 °C until further analysis.

Preparation of uterine secretion metabolites

The collected samples were thawed on ice, and 20 μL of the sample was extracted with 120 μL of pre-cooled 50% methanol, vortexed for 1 min, and incubated at 25 °C for 10 min. The extraction mixture was then stored overnight at -20 degC. After centrifugation at 4000 xg for 20 min, the supernatants were transferred into new 96-well plates. The samples were stored at -80 degC before liquid chromatography-mass spectrometry (LC-MS) analysis. In addition, pooled quality control samples were prepared by combining 10 μL of each extraction mixture.

UPLC-QTOF-MS analysis

All samples were analyzed with an LC-MS system following the manufacturer's instructions. First, all chromatographic separations were performed using a UPLC system (SCIEX, Framingham, MA, USA). An ACQUITY UPLC T3 column (100 × 2.1 mm, 1.8 μm, Waters, Milford, MA, USA) was used for reverse-phase separation. The column oven was maintained at 35 °C. The flow rate was 0.4 mL/min, and the mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). Gradient elution conditions were set as follows: 0–0.5 min, 5% B; 0.5–7 min, 5% to 100% B; 7–8 min, 100% B; 8–8.1 min, 100% to 5% B; 8.1–10 min, 5% B. The injection volume for each sample was 4 μL.

A high-resolution tandem mass spectrometer TripleTOF5600plus (SCIEX) was used to detect the metabolites eluted from the column. The QTOF was operated in both positive and negative ion modes. The curtain gas was set to 30 PSI, ion source gas 1 was set at 60 PSI, ion source gas 2 was set at 60 PSI, and the interface heater temperature was 650 °C. For positive ion mode, the ionspray voltage floating was set to 5000 V. For negative ion mode, ionspray voltage floating was set to -4500 V. MS data were acquired in information-dependent acquisition mode. Dynamic exclusion was set to 4 s. During acquisition, the mass accuracy was calibrated for every 20 samples. Furthermore, to evaluate the stability of LC-MS throughout the acquisition, a quality control sample (pool of all samples) was acquired after every 10 samples.

Statistical analysis and metabolic pathway analysis

XCMS software (Scripps Research Institute, La Jolla, CA, USA) was used to extract and process the characteristic peaks of the original data obtained by LC-MS, the extracted substances were added, and the ions were annotated using a camera. Through principal component analysis, the dimension of the data was reduced, and the overall distribution trend was previewed. Orthogonal partial least squares discriminant analysis was used for modeling the data. Based on a minimum fold change ([?]2), analysis of variance p-value ([?]0.05), and variable projection importance value calculated in the orthogonal projections to latent structures discriminant analysis (OPLS-DA) model ([?]1), the differential metabolites were screened. Candidate markers were annotated with metabolites using the HMDB, KEGG, and other databases. The pathways of different metabolites were further screened by enrichment analysis and topological analysis, and the key pathway with the highest correlation with the metabolite differences was found.

Results

Metabolite profiling of uterine secretion samples

In this study, UPLC-QTOF-MS was used to investigate the metabolite profiles of uterine secretion samples collected from healthy cows and those with clinical endometritis. Positive and negative total ion chromatograms of uterine secretions collected from healthy and clinical endometritis subjects are shown in Figure 2. The results revealed differences between the uterine secretion metabolomes of healthy and clinical endometritis groups.

Metabolomics data analysis

To visualize metabolic differences between the healthy and clinical endometritis groups, unsupervised principal component analysis (PCA) was performed based on the metabolome generated in the positive (Figure 3) and negative ion chromatograms (Figure 4). PCA can be used for the preliminary analysis of samples. Although there were differences among the various components in the PCA score plots, they were difficult to distinguish. Data analysis showed that the individual differences among dairy cows in the same group and other factors produced large intragroup variations. OPLS-DA is a supervised discriminant analysis statistical method for reducing such intragroup differences, enlarging intergroup differences, eliminating the influence of irrelevant factors on experimental data, and effectively predicting different samples. The data were processed by supervised OPLS-DA to identify differential metabolites. The parameters R^2Y and Q^2 were above 0.80 in the two groups, indicating that the model had reasonable interpretation and prediction ability, and the clustering separation effect of samples was noticeable.

Differential metabolites identification

Among the 5566 positive ion and 5983 negative ion metabolites in the uterine secretion of cows, the first principal component variable projection importance >1, difference multiple (FC) > 2 or < 0.5, and $p < 0.05$ were selected as thresholds for the screening of significant metabolites (Table 1). In Figure 4, the abscissa represents the logarithm of the differential multiple of differential metabolites $\log_2$(FC), and the vertical axis represents the negative logarithm of the p-value log10 (p-value), black represents the metabolite with no significant difference (none), red represents upregulated metabolites (up), and green represents downregulated metabolites (down). Figure 5 shows metabolites with significant differences between uterine secretions from healthy cows and those with clinical endometritis, which can be used as the basis for functional analysis of metabolic pathways. Differential metabolites between the uterine secretion metabolomes of healthy versus clinical endometritis samples are listed in Supplemental Figures S1 and S2.

Metabolic pathway analysis

MetaboAnalyst 4.0 was used to investigate metabolic pathway differences between healthy cows and those with clinical endometritis. The major metabolic pathways included those directly involved in lipid and protein metabolism. Ten metabolic pathways showing apparent variations between healthy and clinical endometritis samples are shown in Table 2.

Discussion

Bovine endometritis has received wide attention, as it causes major economic losses to dairy farming. Therefore, accurate and rapid diagnosis of endometritis and effective control measures are crucial to the industry. Previous studies have used metabolomics techniques to identify serum markers of purulent vaginal secretions and subclinical endometritis (Pascottini et al., 2020). In this study, we used UPLC-QTOF-MS to generate and compare the uterine secretion metabolite profiles of healthy cows and those with clinical endometritis. The PCA results revealed differences between the uterine secretion metabolite profiles of the groups. Using OPLS-DA and t -test, we identified candidate cow biomarkers and pathways linked with the endometritis disease status.

Specifically, several metabolic differences were observed in uterine secretion samples collected from endometritis cows compared to healthy cows. There were significant differences in protein and lipid metabolism between the healthy cows and those with clinical endometritis. As the endometritis developed, fewer metabolites of carbohydrate and energy metabolism were detected in the clinical endometritis cow uterine secretions, possibly because of consumption of these metabolites by the pathogen.

The potential biomarkers involved in amino acid metabolism are glutamine, L-glutamate, arginine, and phenylalanine. Glutamine is a nonessential amino acid but has many important physiological functions, such as regulating the metabolism and immune state of the body, protecting the structure and function of the intestinal barrier, and improving the antioxidant capacity of the body (Yeon-Kyung et al., 2018). Under the action of glutamine synthetase, glutamic acid is converted into glutamine, which can be metabolized into glutamic acid. Glutamic acid is a glycogenic amino acid converted into α-ketoglutarate by glutamate

dehydrogenase, which is then converted into malic acid to participate in gluconeogenesis to provide energy for the body (Castell et al., 1994). However, some studies showed that L-glutamic acid is the most important excitatory neurotransmitter in the central nervous system, and a large amount of glutamic acid can cause excitotoxicity, leading to swelling, apoptosis, and necrosis of neurons (Kamel et al., 2018; Kim et al., 2018). Additionally, glutamate can reduce the ratio of Bcl-2 to Bax levels and induce apoptosis (Yamagata et al., 2008). Arginine plays an essential role in hormone secretion, endothelial function, and immunity (Sakari et al., 2015). Under the action of nitric oxide synthase, arginine is converted into citrulline and nitric oxide. Alternatively, it decomposes into urea and ornithine. Ornithine produces proline under the action of pyrroline-5-carboxylic acid reductase. Proline can be used as a free radical scavenger or converted into glutamic acid (Yoshimi et al., 2016). However, high doses of arginine can reduce the synthesis of polyamines and inhibit the synthesis of nucleic acids and proteins. Arginine is the precursor of nitric oxide (NO). After activation of nitric oxide synthase, arginine is converted into NO with the participation of NADPH. Excessive NO release can cause excessive telangiectasis, local inflammatory diffusion, and tissue oxygen utilization reduction, resulting in tissue damage (Jun et al., 2010; Sakuma et al., 1988). Under normal physiological conditions, phenylalanine is hydroxylated to tyrosine, and a transamination reaction produces a small amount of phenyl pyruvic acid. However, under pathological conditions, the conversion of phenylalanine to tyrosine is limited, and instead, it reacts with ketoglutarate to produce phenyl pyruvic acid to result in the accumulation of phenylpyruvate *in vivo* . Excessive accumulation of phenylpyruvate typically causes damage to the body.

Pyruvate metabolism in cows with clinical endometritis was also altered; the lactic acid and succinic acid contents were increased. The increase in lactate in the uterine secretions of cows with clinical endometritis may result from mitochondrial dysfunction and obstruction of cell respiration due to inflammation in the uterus. To meet energy requirements, anaerobic glycolysis increases, leading to lactic acid accumulation (Okorie et al., 2011). Succinic acid, a metabolite of innate immune signaling, can increase the production of IL-1β via the transcription factor hypoxia-inducible factor-1α and amplify the inflammatory effect (Tannahill et al., 2013). The increase in the succinic acid content indicates the development of inflammation.

The potential biomarkers involved in lipid metabolism are linoleic acid, arachidonic acid, lysophosphatidyl cholines, and palmitic acid. Arachidonic acid and linoleic acid are ω-6 polyunsaturated fatty acids, which are biomarkers of various cancers (Ip et al., 1999). Leukotriene, the metabolite of arachidonic acid, mediates inflammatory reactions (Fordhutchinson, 1985). Palmitic acid is the main component of saturated fatty acids in palm oil and has been shown to be associated with metabolic syndrome, cardiovascular disease, neurodegenerative disease, and inflammation (Ebbesson et al., 2015; Warensjö et al., 2005). The levels of linoleic acid, arachidonic acid, and palmitic acid in the clinical endometritis group were higher than those in the healthy group, indicating uterine inflammation.

Glycerin phospholipids are not only important components of the cell membrane but also are closely related to inflammation (Yuan et al., 2019). Lysophosphatidyl cholines (lysoPCs), as chemotactic mediators, can participate in the inflammatory process by modifying immune cell activation by specific G protein-coupled receptors (Gregor et al., 1998). When the lysoPC content is excessive, the cell membrane is damaged and further leads to disordered phospholipid metabolism. Some studies have suggested that some PCs are important biomarkers of inflammation because their levels reliably match the degree of inflammation (Song et al., 2016). LysoPC is a type of bioactive lysophospholipid which is a key signal molecule in cell and tissue metabolism and participates in plasma membrane formation, cell growth and death, and inflammatory reactions. LysoPC is also a new class of inflammatory lipids, which can combine and share metabolic pathways and regulatory mechanisms with thromboxanes, leukotrienes, and prostaglandins (Hideo et al., 2013; Seastou et al., 2013). In uterine secretions, lysoPC 16:0, lysoPC 18:2, lysoPC 17:0, and lysoPC 18:1 were identified as potential regulators of glycophospholipid metabolism. Furthermore, in dairy cows with clinical endometritis, defects in glycerin phospholipid metabolism, fatty acid metabolism, linoleic acid metabolism, and α-linolenic acid metabolism were observed.

We observed an increase in small peptides in uterine secretion samples from cows with clinical endometritis. The increase in small peptides (e.g., Val-Val-Val, Trp-Glu, Phe-His, Tyr-Leu, Trp-Ser, Gln-Phe, and others)

may be due to the activities of proteolytic enzymes of endogenous or bacterial origin, or both (Moussaoui et al., 2002; Wedholm et al., 2008). Thus, the uterine secretion peptide profiles may serve as interesting metabolite biomarkers for diagnosing bovine endometritis; however, further experimental validation is necessary.

Compared with healthy cows, the levels of coumaric acid and benzoic acid, both metabolites of phenylalanine metabolism, were decreased in clinical endometritis uterine secretions. Benzoic acid, a precursor of hippurate, can inhibit the growth of coliforms (Knarreborg et al., 2002). Coumaric acid exerts essential antioxidant, cardio-protective, anti-malarial, anti-mutagenic, anti-platelet, anti-inflammatory, and immune-modulatory effects (Jiao et al., 2018; Pei et al., 2016; Pragasam et al., 2013). Notably, the uterine secretions of healthy dairy cows contain large amounts of benzoic acid and coumaric acid, protecting the uterus from invasion by *Escherichia coli* , and inducing anti-inflammatory effects. We also observed a significant decrease in equol content. Equol is the metabolite obtained from soybean isoflavones under the action of the intestinal microflora, which has hormone-like effects and antioxidant and immune regulatory functions (Sugiyama et al., 2013). Compared with healthy cows, the content of equol in the uterine secretion of cows with clinical endometritis is lower, indicating that the reproductive tract is affected.

Apart from confirming some known bovine endometritis biomarkers (e.g., cholesterol, salivary amylase, cortisol, uric acid, adenosine deaminase, and acetylcholinesterase), we identified new metabolites such as lactic acid, succinic acid, glutamine, palmitic acid, and benzoic acid, which varied considerably between the healthy and endometritis groups. This study improves the understanding of the pathobiology of endometritis and provides tools for diagnosing endometritis.

However, one limitation of this study was the relatively small sample size. Further experimental validation of our findings is needed prior to the application of this method to farm conditions. Different microbial infections of the uterus may significantly impact uterine secretion metabolism. A more comprehensive multi-omics analysis can further reveal the pathological and biological reactions of endometritis.

Conclusions

We used UPLC-QTOF-MS technology to comparatively analyze the uterine secretion metabolome of samples collected from healthy cows and those with clinical endometritis. We identified numerous differential metabolites, including glutamic acid, benzoic acid, equol, phenylalanine, lysoPCs, palmitic acid, lactic acid, and succinic acid. Further experiments are required to investigate the potential use of these differential metabolites as biomarkers for endometritis diagnosis, particularly for defining the disease stages. Moreover, this study demonstrates that implementing UPLC-QTOF-MS technology in uterine secretion metabolome analysis is a promising approach for reliably diagnosing bovine endometritis.

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Conflict of Interest

None

Data availability

All data, models, or code that were generated or used during the study will be available from the corresponding author on request.

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Figure legends

Figure 1. Diagnosis of cow endometritis. A. Vaginal discharge score. B. Chemical examination of endometritis. -:healthy,+:Endometritis.

Figure 2. Total ion chromatogram. A. Total ion chromatogram of positive ions. B. Total ion chromatogram of negative ions.

Figure 3. Metabolomics data analysis of positive ions. A. Principal component analysis of positive ions. B–C. Schema sorting verification of positive ions.

Figure 4. Metabolomics data analysis of negative ions. A. Principal component analysis of negative ions. B–C. Schema sorting verification of negative ions.

Figure 5. Volcanic maps of differential metabolites of positive ions (A) and negative ions (B).

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