Revised

**Metabolic profiles using ¹H-NMR spectroscopy in postpartum dairy cows with
ovarian inactivity**

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Abstract

To understand the differences in metabolic changes between cows with ovarian inactivity and estrus cows, we selected cows at 60–90 d postpartum from an intensive dairy farm. According to clinical manifestations, B-ultrasound scan, rectal examination, 10 cows were assigned to the estrus group (A) and 10 to the ovarian inactivity group (B). All plasma samples were analyzed by ^1H -NMR spectroscopy to compare plasma metabolomic profiles between the groups. We used multivariate pattern recognition to screen for different metabolites in plasma of anestrus cows. Compared with normal estrous cows, there were abnormalities in 12 kinds of metabolites in postpartum cows with ovarian inactivity ($|r| > 0.602$), including an increase in acetic acid ($r = -0.817$), citric acid ($r = -0.767$) and tyrosine ($r = -0.714$), and a decrease in low-density lipoprotein ($r = 0.820$), very low density lipoprotein ($r = 0.828$), lipids ($r = 0.769$), alanine ($r = 0.816$), pyruvate ($r = 0.721$), creatine ($r = 0.801$), choline ($r = 0.639$), phosphorylcholine ($r = 0.741$) and glycerophosphorylcholine ($r = 0.881$). These metabolites were closely related to abnormality of glucose, amino acid, lipoprotein and choline metabolism, which may disturb the normal estrus. The decrease in plasma creatine and the increase in tyrosine were new changes for ovarian inactivity of postpartum cows. The decrease in plasma creatine and choline and the increase in tyrosine and p-hydroxyphenylalanine in cows with ovarian inactivity provide new directions for research on the mechanism of ovarian inactivity in cows.

Key words: Dairy cows; Ovarian inactivity; Nuclear magnetic resonance technology; Plasma differential metabolites

1. Introduction

As the number of large intensive dairy farms, and yield of dairy cows have increased, so too has the incidence of reproductive disorders in dairy cows [1, 2]. In China, the morbidity from reproductive disorders is 20–40%, with anestrus in 26.68%. Therefore, there is an urgent need to resolve the problem of anestrus in postpartum cows because of its influence on the reproductive performance of dairy cows.

Ovarian inactivity is a common type of anestrus in postpartum cows. This condition is characterized by growth of follicles only to the stage of follicular wave emergence, that is, up to ~8 mm diameter, after which, growth stops [3]. At 40d postpartum, when uterus and ovary recover to normal physiology, there are no substantial changes in the follicular structures, accompanied by characteristic absence of a corpus luteum or cystic follicular structures [4].

Follicular growth needs various metabolites and environmental factors. This potential carry-over effect of adverse conditions during oocyte growth and maturation on further fertility outcome was first proposed by Britt in 1992 [5]. Britt hypothesized that the developmental competence of the oocyte and the steroidogenic capacity of the follicle in high-yielding dairy cows are determined by their biochemical environment during a long period (up to 80 days) of follicular growth before ovulation [5]. Follicular growth is sensitive to metabolic signaling. Some research has indicated that changes in estradiol, growth hormone, insulin-like growth factor-I, insulin, alanine and glutamine can affect follicular growth and maturation [6]. Previous studies have not investigated the overall metabolic changes in healthy cows and cows with ovarian

inactivity.

In recent years, as metabonomics technology has improved gradually, liquid chromatography tandem mass spectrometry, gas chromatography tandem mass spectrometry, and nuclear magnetic resonance (NMR) have been used to screen for diagnostic biomarkers of milk fever [7] and ketosis[8], and to compare the pathogenic mechanism of type I and II ketosis[9]. Postpartum ovarian inactivity in cows is affected by many factors, and the metabolic process is complicated and dynamic. Traditional one-dimensional research cannot describe systematically and objectively the metabolic changes in cows with ovarian inactivity. Therefore, metabolomics ¹H-NMR technology was used to analyze the different metabolites in plasma of cows with ovarian inactivity and plasma of estrus cows in our study. We aimed to clarify the overall metabolic changes in cows with ovarian inactivity, provide a theoretical basis for research into the mechanisms, prevention and cure of ovarian inactivity.

2. Materials and methods

2.1 Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental animals were treated according to the International Guiding Principles for Biomedical Research Involving Animals.

All cows were obtained from an intensive dairy farm in China. All diets were in accordance with the Chinese standards for cattle breeding. Cows were housed in intensive farming conditions with continuous access to fresh water, and were milked 3

times daily. Two hundred and six healthy Holstein cows with similar age and parity were selected as experimental animals. They were tracked to 60–90 days postpartum to observe the symptom of estrus and determine the follicle size by rectal examination and B ultrasound examination.

Normal estrus cows: spontaneous estrus at 60–90 d postpartum with obvious estrous signs. Rectal examination and B-ultrasound showed that there were no uterine and ovarian abnormalities and mature ovulatory follicles were present. The size of follicle was 15–20 mm by B-ultrasound.

Ovarian inactivity cows: a cow was considered to have ovarian inactivity when a follicular structure <8 mm was detected in two consecutive examinations, in the absence of a corpus luteum or cyst and no estrus signs were noted during the 7-day period between the examinations.

Ten estrus cows without other diseases were selected as the estrus group (A) and 10 cows with ovarian inactivity without other diseases were selected as the ovarian inactivity group (B).

2.2 Sample collection

Blood were taken in the 45-60 days of postpartum, when the estrus cows were in the stage of ovulation or will to be ovulation condition. At the same time, the blood samples of the cows which in the same postpartume days but had no follicle structure or the follicle size less than 8.5mm were taken, if these cows have no follicle growth in the 60-90 days postpartum,the samples were as expriment group. If the cows estrus latter, the sample will be disgard.Samples of 10 estrus cows and 10

ovarian inactivity cows were taken finally.

Ten milliliters of blood was taken from the jugular vein of the 20 cows. The blood was placed in an anticoagulant tube with 3–5 drops of heparin sodium, and blood and anticoagulant were mixed evenly. Blood samples were centrifuged at $1500 \times g$ for 5 min, the plasma were transferred to a 1.5-mL microcentrifuge tube, then the supernatant was centrifuged at $12,000 \times g$ for 5 min, and 500 μ L plasma was transferred to a 1.5-mL microcentrifuge tube, and stored at -80°C .

2.3 Sample preparation

Before ^1H -NMR detection, all serum samples were thawed at room temperature. Aliquots of 200 μ L were placed in 1.5-mL microcentrifuge tubes combined with 400 μ L 99.8% D_2O buffer salt solution (pH 7.0) containing 0.2 mol/L Na_2HPO_4 and 0.2 mol/L NaH_2PO_4 , 0.05% (w/v) sodium 3-(trimethylsilyl) propionate-2,2,3,3,- d_4 (TSP). The mixture was centrifuged at $12,000 \times g$ for 20 min at 4°C , and 550- μ L aliquots were taken into 5-mm NMR tubes (Wilmad) for detection.

2.4 ^1H -NMR spectroscopy

The NMR tubes that contained the 500- μ L samples were placed under the signal acquisition probe of a 600-MHz digital NMR spectrometer (AVANCE III 500MHz; Bruker, Switzerland). The NMR spectra were recorded using a Call–Purcell–Meiboom–Gill (CPMG) sequence, with 64 timed scans and a 10-ms total relaxation period (transverse and longitudinal relaxation) to inhibit the protein signal. The free induction decay (FID) signal was measured following transverse relaxation. All FID signals were multiplied by the exponential window function with a

1-Hz broadening factor before Fourier transformation to improve the signal-to-noise ratio, then the ^1H NMR spectra were obtained.

2.5 Preprocessing of ^1H -NMR spectra

To obtain meaningful information from a mass of spectral data, dimensionality reduction ideology should be used to pretreat the data. The ^1H -NMR spectra were imported into Topspin version 3.0 software (Bruker, Ettlingen, Germany), and manually corrected, adjusting the baseline phase and phase position. The ^1H -NMR spectra were referenced to an internal α glucose resonance at δ 5.23. The spectral area within each bin was integrated. The bin size of δ 0.002 was chosen as the primary chemical shift bin width to avoid the encompassment of several peaks and to have clear peak multiplicities, which facilitated identification of metabolites. The water resonance (5.17–4.47 ppm) and urea resonance (6.60–5.42 ppm) regions were excluded to eliminate baseline effects. The spectra were transformed into ASCII files using MestReNova version 8.0.1 software (Mestrelab Research, Santiago de Compostela, Spain), and saved in plain text format, which included the chemical shift data for each NMR spectral peak.

2.6 Identification of compounds

^1H -NMR spectral data were imported into the Chenomx NMR version 7.5 software suite (Chenomx, Edmonton, Canada). Compounds in Chenomx were chosen for automatic identification by comparison with the library of the Chenomx software. The displacement and peak were recorded. The compounds that were well matched were chosen and compared with the Human Metabolome Database

(<http://www.hmdb.ca>), the Madison–Qingdao Metabolomics Consortium Database (<http://mmcd.nmrfa.wisc.edu/>), and the Kyoto Encyclopedia of Genes and Genomes (<http://www.kegg.jp>). Detailed information and metabolic pathways of compounds were found.

2.7 Multivariate statistical analysis

NMR data were imported into SIMCA-P version 13.0 software (Umetrics, Umea, Sweden). Multivariate analysis included unsupervised principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

PCA was used to assess variance among predefined groups and to remove irrelevant factors. OPLS-DA was used to maximize the covariance between the measured data (X variable, peak intensities in NMR spectra) and the response variable (Y variable, predictive classifications) and to remove simultaneously uncorrelated variations between the X and Y variables or X variables that were orthogonal to Y variables. OPLS-DA was based on a unit variance scaling strategy. A 10-fold cross-validation was used to obtain Q^2 and R^2 values, which represented the predictive ability of the model and the explained variance, respectively. Sensitivity, specificity, and classification rates (i.e., percentage of samples correctly classified), which were obtained within a 10-fold cross-validation of OPLS-DA models among groups, were calculated.

Correlation coefficients of metabolites were obtained by OPLS-DA, and the metabolites with statistical significance were analyzed further. One-way analysis of variance was performed using SPSS version 17.0 (IBM, Armonk, NY, USA). The

level of statistical significance was set at $P < 0.05$ for the identification of differentially expressed metabolites.

2.8 Analysis of differences in metabolic pathway and interaction network construction

Human Metabolome Database (HMDB, <http://www.hmdb.ca>) and KEGG were selected to make a search of well-matched compounds, and detailed information and metabolic pathways of the different metabolites was obtained.

3. Results

The age, parity and body condition score (BCS) of estrus and ovarian inactivity cows are listed in Table 1, which shows that the two groups of cows have similar age, parity and BSC.

Table 1. Clinical data of estrus cows (10) and ovarian inactivity cows(10) (Mean $\pm$ SD)

Index	Estrus (A)	Ovarian inactivity (B)	P value
Age	3.65 $\pm$ 1.41	3.46 $\pm$ 1.26	0.78
Parity	2.20 $\pm$ 1.03	2.20 $\pm$ 1.03	1.00
BCS	2.85 $\pm$ 0.29	2.78 $\pm$ 0.14	0.48

BCS = body condition score; $P > 0.05$ indicates no significant difference between two groups.

Representative ^1H NMR spectra of plasma samples from the estrus and ovarian inactivity groups are shown in Fig. 1. All signal peaks were contained within 0.5–8.5 ppm (where ppm represents the chemical shift of the compound), and some of the peaks were expanded 32 times for convenient observation. Data obtained from the ^1H

NMR spectra were analyzed; 16 differential compound were identified (Table 2), and the data were subsequently analyzed using multivariate statistics.

Fig. 1. 600 MHz ^1H NMR spectra of representative samples from groups A (estrus,10) and B (ovarian inactivity,10).

Table 2. Names and abbreviations of 22 key differential compounds from groups A (estrus,10) and B (ovarian inactivity,10).

No.	Abbreviation	Name
1	Val	Valine
2	Leu	Leucine
3	Ile	Isoleucine
4	3-HB	3-Hydroxybutyrate
5	Ala	Alanine
6	Lys	Lysine
7	Arg	Arginine
8	Glu	Glutamate
9	Crea	Creatine
10	LDL(L1,L3)	Low-density lipoprotein
11	VLDL(L2,L4,L5)	Very-low-density lipoprotein
12	Lipid (L6,L7,L8,L9)	Lipid
13	NAG	N-acetyl glycoprotein signals
14	Aspg	Asparagine
15	PC	Phosphate choline
16	GPC	Glycerophosphate choline

Principal component analysis (PCA) was performed first to examine intrinsic patterns in the data set and to gain an overview of variation among the groups; however, PCA could not sufficiently separate the two groups (Fig. 2). OPLS-DA was then performed to gain better group separation and to reveal differential metabolites between the two groups. In the OPLS-DA score plot (Fig. 3), the two groups were well separated ($R^2Y = 0.35$, $Q^2Y = 0.563$). By assessing the coefficient plots of OPLS-DA score plot and color-coded loading plots, the main metabolites contributing

to the separation of the two groups are shown in Table 1. Marked differences were found between the two groups.

Fig. 2. PCA scores plot based on ^1H -NMR spectra of plasma of dairy cows obtained from Groups A (estrus,10) and B (ovarian inactivity,10).

Note: Group A (estrus): box (■); Group B (ovarian inactivity): dot (●); $t[1]$ and $t[2]$ represent first two principal components; R^2X and Q^2 represent the explicable variables and the predictable degree of the model, respectively.

Fig. 3. OPLS-DA scores plots (left panel) derived from ¹H-NMR spectra of dairy cows plasma and corresponding coefficient loading plots (right panel) obtained from Groups A (estrus,10) and B (ovarian inactivity,10).

Note: Left panel :Group A (estrus): box (■); Group B (ovarian inactivity): dot (●); t[1] and t[2] represent first two principal components; R²X and Q² represent the explicable variables and the predictable degree of the model, respectively.

Right panel :The map shows the significance of metabolite variations between the two classes. Peaks in the positive direction indicate metabolites that were more abundant in Group A. Consequently, metabolites that were more abundant in Group B are presented as peaks in the negative direction.

Twelve differential metabolites were screened from the plasma of the ovarian inactivity group. The concentration of acetic acid, citric acid and tyrosine increased, and the concentration of plasma low density lipoprotein (LDL), very low density lipoprotein (VLDL), lipid, alanine, pyruvic acid, creatine, choline, phosphorylcholine and glycerophosphoryl choline was decreased significantly. An interaction network was formed by 9 metabolites. These metabolites were formed by glycerol phospholipid metabolic pathway; glycine, serine and threonine metabolic pathway, citrate cycle pathway, and glycerophospholipid metabolic pathway. Table 3 shows the 12 differential metabolites and 9 major pathways in plasma of dairy cows with estrus and ovarian inactivity.

Table 3. 12 differential metabolites and major pathways in plasma of dairy cows with ovarian inactivity(10) compared to cows with estrus(10)

No.	Metabolites	KEGG ID	Chemical shift (ppm)	Correlation coefficients (r ^a)	Change trend	Pathway
1	Alanine	C01401	1.48(d)	0.816	↓	Cyano-amino acid metabolism
2	Acetic acid	C00033	1.91(s)	-0.817	↑	Pyruvate metabolism and glycolysis or gluconeogenesis
3	Pyruvate	C00022	2.36(s)	0.721	↓	Glycine, serine and threonine metabolism and citrate cycle
4	Citric acid	C00158	2.55(d)	-0.767	↑	Citrate cycle (TCA cycle)
5	Creatine	C00300	3.03(s)	0.801	↓	Glycine, serine and threonine metabolism
6	Choline	C00114	3.20(s)	0.639	↓	Glycerophospholipid metabolism and glycine, serine and threonine metabolism
7	Choline phosphate	C00588	3.22(s)	0.741	↓	Glycerophospholipid metabolism
8	Glycerol phosphate choline	C00670	3.23(s)	0.881	↓	Glycerophospholipid metabolism
9	Tyrosine	C00082	6.89(d)	-0.714	↑	Ubiquinone and other terpenoid-quinone biosynthesis and phenylalanine, tyrosine and tryptophan biosynthesis
10	Lipid (LDL)	-	0.86(mb)	0.820	↓	-
11	Lipid (VLDL)	-	1.36(m)	0.828	↓	-
12	Lipid	-	5.30(m)	0.769	↓	-

Note: ^aCorrelation coefficients, positive and negative signs indicate positive and negative

correlation in the concentrations, respectively. The correlation coefficient of $|r| > 0.602$ was used as the cutoff value for statistical significance based on the discrimination significance at the level of $P=0.05$ and df (degree of freedom) = 10 (A vs. B). “↓” Compared to the estrus cows, the ovarian inactivity cows had down-regulated expression; “↑” Compared to the estrus cows, the ovarian inactivity cows had up-regulated expression. VLDL = Very low density lipoprotein, LDL = Low density lipoprotein, KEGG= Kyoto Encyclopedia of Genes and Genomes.

4. Discussion

Differential plasma metabolites of cows with ovarian inactivity were selected by ^1H -NMR in this study. The concentrations of 12 plasma metabolites differed between estrus cows and ovarian inactivity cows. Acetic acid, citric acid and tyrosine were increased, whereas LDL, VLDL, lipids, alanine, pyruvic acid, creatine, choline, phosphorylcholine and glycerophosphorylcholine were decreased. Abnormal concentrations of these metabolites could be summarized into four categories: glucose metabolism, amino acid metabolism, lipoprotein metabolism and choline metabolism.

The database search showed that 9 different metabolites were matched, which belonged to the citric acid cycle, glycine, serine and threonine metabolism pathway, and glycerol phospholipid metabolism pathway. The most relevant metabolic differences between estrus and ovarian inactivity cows are described in Fig. 4. The grey labels indicate the different metabolites that were detected, and the others represent the upstream and downstream metabolites of these different metabolites. These 9 metabolites interact with each other, leading to formation of a metabolic interaction network diagram.

Fig. 4. Summarized different metabolic pathways of Groups A (estrus,10) and B (ovarian inactivity,10).

Note: Metabolites in light grey represent a higher concentration in Group B(ovarian inactivity) compared with Group A(estrus), and those in dark grey had a lower concentration in Group B (ovarian inactivity)compared with Group A(estrus). The corresponding metabolic pathway is highlighted.

In the differential metabolites, compared with the estrus group, the concentration of acetic acid and citric acid was higher in the ovarian inactivity group, and the concentration of pyruvate was lower. Three metabolites were involved in glucogenesis and the citric acid cycle, which are involved in glucose metabolism. In the citric acid cycle, the end product of glycolysis, pyruvic acid, is decarboxylated into acetyl CoA and then it passes through this pathway to produce citric acid. Acetic acid and acetyl CoA are transformed with each other, and pass through the citric acid

cycle to produce citric acid. Another route of pyruvic acid metabolism is transformation into amino acids, such as serine and alanine, which then pass into the glycine, serine and threonine metabolism pathway [10]. The decrease of pyruvate may be due to increased consumption of pyruvate to produce acetyl CoA and citric acid, and citric acid to produce energy, which causes accumulation of acetic acid and citric acid [11]. Nandi et al. investigated the effect on follicular growth of adding different concentrations of pyruvate, glucose and lactate to follicular fluid. Culture of oocytes and follicular somatic cells in medium containing pyruvate alone resulted in cell growth comparable to that in medium containing glucose alone or physiological concentrations of glucose, lactate and pyruvate in combination [12]. This indicated that pyruvate played an important role in energy production in follicular growth. In our study, the concentration of pyruvate decreased and the downstream metabolites of pyruvate increased. This indicates that gluconeogenesis is used for maintenance of normal physiology in cows, and the pyruvate may be used by cells to provide energy to meet follicular growth.

Alanine is a nonessential amino acid that is formed by transamination of pyruvic acid[13]. As pyruvate concentration decreases, so too does formation of alanine. That is to say, pyruvate increases citric acid generation and reduces conversion to alanine. The results of pathway analysis show that another way of pyruvate metabolism is getting into glycine, serine and threonine metabolism to product serine. The decrease in pyruvate may cause a decrease in glutamic acid and serine. Amino acids play an important role in follicular growth. Sinclair et al. indicated that alanine and glycine

are the most abundant amino acids in follicular fluid. These two types of amino acids have many functions related to cell growth, except protein biosynthesis, and they could be involved in nucleotide biosynthesis and osmoregulation [14]. Matobas et al. compared the metabolite differences in follicular fluid during normal growth and after follicular growth had stopped. They found that the concentration of alanine, glycine and glutamic acid had a positive correlation with follicular growth [15]. In our study, the concentration of alanine decreased, which may have affected metabolism of serine and glutamic acid. Because the two group cows received the same diet, the level of amino acid taken may be in the same level. The decrease of amino acid in ovarian inactivity indicates cows in ovarian inactivity would have a higher requirement for amino acid. As an essential amino acid, lysine cannot be synthesized *in vivo*, and must be taken up by the diet [16]. The increased lysine concentration in the present study may have been due to inhibition of utilization and conversion, which led to accumulation in the body. Lysine is a ketogenic amino acid that is involved in acetone body generation, therefore, increased plasma lysine content suggests that the ketogenic process has been hindered. Issues of utilization and metabolism of lysine need to be addressed. This not only wastes dietary lysine, but also suggests that it is not playing its proper role.

Abnormal lipoprotein metabolism included abnormal of LDL, VLDL and lipid metabolism. Previous studies pointed out that, when cows have ketosis, there is insufficient glucose, fat and protein mobilization is accelerated to maintain energy needs, and levels of LDL and VLDL are decreased[17, 18]. VLDL is an important

transport protein that can transport glycerin trilaurate, and the decrease in plasma VLDL suggests that most VLDL accumulated in the liver to remove excess triglyceride produced by fat mobilization.

Among the differential metabolites, the concentration of creatine, choline, phosphocholine and glycerophosphocholine was decreased in ovarian inactivity cows. These four metabolites were involved in the pathway of glycerophospholipid metabolism. About 60–70% of creatine in muscle is in the form of creatine phosphate, so myofibrils produce enough energy for muscle contraction [19]. Our results showed that creatine content decreased in cows with ovarian inactivity, which may have been related to the energy consumption in inactivity cows increased. Choline is a component of all steroid hormones (including sex and adrenocortical hormones), therefore, a decrease in choline concentration will lead to a concomitant decrease in the level of sex hormones, which indicates that choline is an important biological marker in cows with ovarian inactivity. In the glycerophospholipid metabolism pathway, phosphocholine and glycerophosphocholine are transformed into choline. As an important energy substrate, choline can provide energy by increasing glucose concentration[20]. Creatine can provide energy directly. Creatine and choline have an effect on methyl glycosine, but a decrease in these two substrates has no effect on methyl glycosine. This suggests that methyl glycosine was not the main metabolic difference between cows with ovarian inactivity and estrus cows, creatine and choline synthesis was disrupted, and metabolism of creatine and choline was normal and it is a problem of available amount or synthesis. This suggests that cows with ovarian

inactivity have insufficient creatine and choline, or that their synthesis is inhibited. However, insufficient creatine and choline could just be an individual difference among some cows, because this condition did not occur in many cows. Therefore, we suppose that some substance or pathway that regulates choline and creatine metabolism is altered in cows with ovarian inactivity.

5. Conclusion

In our study, the differential plasma metabolites of cows with ovarian inactivity were selected by ^1H -NMR technology. Compared with estrus cows, there were abnormalities in 12 plasma metabolites; among which acetic acid, citric acid and tyrosine were increased, and LDL, VLDL, lipids, alanine, pyruvic acid, creatine, choline, phosphorylcholine and glycerophosphorylcholine were decreased. These differences in metabolite concentrations reflect a variety of metabolic disorders, such as abnormal glucose, amino acid, lipid and choline metabolism. The decrease of creatine and choline concentration and the increase of p-hydroxyphenylalanine concentration in cows with ovarian inactivity provide a new direction for research on the mechanism of ovarian inactivity in cows.

6. Acknowledgments

The authors thank the National Science Foundation Committee (31672625), Natural Science Foundation of Heilongjiang Province of China(C2016041) and Heilongjiang Bayi Agriculture University graduate student innovation fund project (YJSCX2015-Y22) for the financial support. Thank the International Science Editing for manuscript editing.

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