Metabolite Comparison between Serum and Follicular Fluid of Dairy Cows with Inactive Ovaries Postpartum

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Abstract

Inactive ovaries (IO) is a kind of postpartum ovarian disease in dairy cows, which sometimes accounts for 50% of ovarian disease, which seriously affects the dairy cows's reproductive efficiency. To explore the metabolic changes in the serum and follicular fluid of dairy cows with IO during lactation, in this study six estrus (E) cows and six IO cows at 50 to 55 days in milk were selected according to B ultrasonic detection and clinical manifestations. The differential metabolites in serum and follicular fluid between the E cows and IO cows were identified by ultra-high-pressure liquid chromatography quadrupole time-of-flight mass spectrometry, combined with multidimensional statistical methods. The result shows that dairy cows with IO were in a subclinical ketosis status (BHBA > 1.20 mmol/L), 14 differential metabolites in the serum of IO cows included 10 increased metabolites and 4 decreased metabolites, while 14 differential metabolites in the follicular fluid of IO cows included 8 increased metabolites and 6 decreased metabolites. These differential metabolites mainly involve 9 metabolic pathways. Among them, the common enrichment pathway of different metabolites in serum and follicular fluid are glycerophospholipid metabolism, pentose and glucuronate interconversions. In conclusion, there are significant differences in the differential metabolites and enrichment pathways between serum and follicular fluid of IO cows. It implies that there are the complex changes in blood metabolism and local follicular metabolism of IO cows with subclinical ketosis, which their interaction relationship needs to be further confirmed.

Introduction

With rapid increase of milk production in dairy cows, the reproductive performance has gradually declined in the past few decades. Cows with high milk production usually have low postpartum reproductive efficiency, which seriously affects the healthy development of the dairy industry (Wang et al., 2014). The reproduction events of dairy cow are long and complex. Preovulatory reproductive failure due to cow metabolic changes in transition period potentially affectes the timing of return to oestrus (Roche et al., 2011). It had been known that inactive ovaries (IO) is a kind of postpartum ovarian disease in dairy cows, which sometimes accounts for 50% of ovarian disease (Roth et al., 2008). For 45–60 days postpartum, cows do not show estrus and there is no corpus luteum on the surface of the ovaries. Follicle waves appear on the surface of the ovaries, but the growth of the follicles stops before the follicles deviate, which is called inactive ovaries (IO) (Butler et al. 2003). Therefore, in the modern intensive dairy farms, prevention and treatment of ovarian inactivity has become an important challenge of reproduction disorders during ealry lactation.

Follicular fluid (FF) is the microenvironment for the development and maturation of oocytes in animals and human. It contains biologically active molecules and proteins that may affect follicular growth and oocyte fertilization and can affect the quality of oocytes, fertilization, and possibly even the development of embryos (Revelli et al. 2009). As the follicle develops, the composition of the FF will change (Spitzer et al. 1996; Ferrazza et al. 2017), proving that it participates in the development of the follicle and oocyte. Its

composition is therefore closely related to oocyte meiosis, ovulation, corpus luteum formation and fertilization (Schweigert et al. 2006). The metabolites contained in FF mainly come from blood and secretions by theca cells, granulosa cells (GCs) and the oocyte (Hennet & Combelles, 2012; Schweigert et al. 2006) included lipids, glucose, proteins, cytokines, steroids, growth factors and peptide hormones (Leroy et al. 2011; Van et al. 2013; Wrenzycki & Stinshoff, 2013). Thus, it is necessary to jointly analyse the similarities and differences between the metabolism of follicular fluid and serum of dairy cows with IO during early lactation.

Metabolomics technology has developed into a mature technology in the past decade, which can directly detect the physiological and pathological states of individuals, provide the most comprehensive and direct characterization, and provide information that can reveal the pathological changes and mechanisms of disease occurrence (Wishart. 2016). Luo Z et al. (2019) reported plasma metabolite changes in cows during parturition, providing new information on new pathways for the initiation of physiological responses under the stress of parturition. Ametaj et al. (2014) used metabolomic methods to reveal changes in plasma amino acid and sphingolipid profiles in transitional diseased cows, providing evidence for selected amino acids as biomarkers of disease or deviation from normal health status. However, so far there have been no reports on metabolic changes between in blood and in FF simultaneously of ovarian diseases in dairy cows during early lactation.

Our purpose is to explore the metabolic changes in blood and follicular fluid of dairy cows with IO through liquid chromatograph-mass spectrometer (LC/MS) technology, and to compare differential metabolites (DMs) of serum and FF of postpartum IO cows by statistical analysis and information biology analysis. It will provide directions for future in-depth research of IO in dairy cows.

Materials And Methods

Sample Collection and Clinical Information

According to the requirements of the Veterinary Medical Ethics Committee of the Ministry of Agriculture of China, this experiment was carried out in a large intensive cattle farm in the central region of Heilongjiang Province. All experiments on animals were conducted according to the standards approved by the Animal Welfare and Research Ethics Committee at Heilongjiang Bayi Agricultural University (No. 20200127) and carried out in compliance with the ARRIVE guidelines. The total mixed ration (TMR) of tested dairy cows complied with the NRC (2001) and with the Chinese Feeding Standard for dairy cows. The composition and chemical components of TMR diet included 1.03 kg cottonseed, 1.50 kg soybean husk, 2.50 kg alfalfa, 1.30 kg soybean meal, 2.00 kg corn flake, 1.00 kg molasses, 25.37 kg silage, and 3.00 kg corn, with a milk net energy of 0.78 Mcal/kg.

Holstein cows were selected as the experimental animals from a large intensive farm in Heilongjiang, China. The BCS (a five-point scale) of 1 (thin) to 5 (obese) points with intervals of 0.25 was used to assess body fat stores (Ferguson et al., 1994). After 50 to 55 d postpartum, the milk yield of cows was

recorded on the same day as blood samples collection. Meanwhile, rectal examination, B-ultrasonic detection and clinical manifestations were implemented to exclude cows with other diseases or clinical abnormalities, and six normal cows with estrus behaviors were selected as an estrus (E) group and six cows with IO were selected as an IO group. The IO cows had no significant fluctuation in the activity of cows at 50 to 55 d postpartum and no clinical estrus by the Afimilk Ranch Management System (Afimilk®3.076, Israel), and no fluid in the uterine cavity, normal ovarian hardness and size, follicle diameter less than 8 mm and follicle enlargement < 3mm within 5d by B-ultrasound examination of endometrial folding (Nelson et al. 2017). In contrast, the E group showed fluctuations in the step count of cows at 50 to 55 d postpartum and clinical manifestations of estrus, such as crawling or moist and swollen mucous membranes of the labia. B-ultrasonography Examination was used to examine the muscularis and intima layers of the cervix, there were obvious boundaries, thickened uterine wall, uneven hardness, fluid in the uterine cavity and ovarian follicles with a diameter of 15 to 20 mm of E cows. The serum samples were collected through the tail vein of cows in the morning before feeding, and centrifuged at 3,000 rpm for 10 min to collect the supernatant into 1.5 mL tube, then recentrifuged at 12,000 rpm for 10 min to collect supernatant to store at -80°C. At 50 to 55 d postpartum, a transvaginal ultrasound guided aspiration technique (Sonosite-Titan, 10 MHz, micro convex probe, USA) was used on the two groups of cows to collect FF after measuring the follicular diameter (Pancarlı et al. 2011), and centrifuged at 12,000 rpm for 15 min to collect the supernatant to stored at -80°C for proteomic analysis (Moore et al. 2017). Clinical information such as milk yield, body condition, age and diseases was collected at the same time by the Afimilk Ranch Management System.

Blood biochemical index detection

Serum biochemical indicators involve in energy metabolism, minerals, and liver function. The energy metabolism indicators include BHBA, NEFA, and Glu(mmol/L). Mineral index includes Ca, P, and Mg (mmol/L). Liver function index include ALT, AST (U/L), and TP (g/L). Serum BHBA using a blood ketone meter and ketosis reagent strips with 93.8% sensitivity, 100% specificity, and a 93.8% Youden index (Yicheng. Beijing. China). Other biochemical parameters were measured using commercial kits (Biosino Biotechnology and Science inc, China).

Sample Pretreatment

The first was to pipette 100 µL of serum or FF sample into a centrifuge tube. add 300 µL of methanol mixture containing the internal standard L-2-chlorophenylalanine 1 µg/mL, vortex and mix for 30 s. It was then placed in an ice water bath and sonicated (Shenzhen Redbond Co., Ltd., PS-60AL) for 10 min and then incubated for 1 hour in a refrigerator at -40°C. The test sample was centrifuged at 12,000 rpm for 15 min at 4°C and finally 60 µL of the supernatant was drawn into a sample bottle for testing on the Q Exactive HFX mass spectrometer (AB Sclex, USA). A quality control sample (QC) was created by taking an equal amount of supernatant from all samples and mixing them together for mass spectrometer detecting.

Detection of Serum and FF by LC/MS

Ultra-high-pressure liquid chromatography (Vanquish, Thermo Fisher Scientific, USA) with a liquid chromatograph column (Waters ACQUITY UPLC BEH Amide, 2.1 mm × 100 mm, 1.7 μm) were used to separate the serum and FF samples from the test cows. The Q Exactive HFX mass spectrometer (AB Sciex, USA) performed primary and secondary mass spectrometry data acquisition using the control software (Thermo, Xcalibur). In NCE mode, voltage was 3.5 kV in positive ion mode or -3.2 kV in negative ion mode. The detailed steps refer to our previous report (Bai et al. 2020).

Differential Screening and Statistical Analysis of Data

The SPSS 23.0 software (IBM, USA) was used for statistical analysis of clinical information and serum biochemical indicators collected from experimental cows via one-way ANOVA. The data is shown as the mean ± standard deviation.

After obtaining the finished metabolomics data, multivariate statistical analysis was performed. The SIMCA software (V15.0.2) was used to perform logarithmic (LOG) conversion and centralization (CTR) format processing on the data and automatic modeling and analysis. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used for the results. Seven-fold cross validation was used to test the model, cross-validation Q² and R²_Y used to determine whether the model is valid and then through a permutation test, the order of the categorical variable Y was randomly changed several times to obtain the R and Q values of the random mode. Finally, a t-test was performed on the metabolomics data to further check that the model was effective. The final selection criteria for DMs was that $P < 0.05$ and the variable projection importance (VIP) of the OPLS-DA model was greater than 1.

Correlation Analysis between DMs and Metabolic Pathway Analysis.

The compounds were screened by the Human Metabolome Database (HMDB) and the Madison Metabolomics Consortium Database (MMCD). MetaboAnalyst 3.0 was used to analyze the DMs selected by multivariate statistical analysis and univariate analysis. Visual analysis of metabolites was carried out using hierarchical clustering and a heatmap. Through the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database, all pathways were selected corresponding to the mapping of DMs, and conduct topological analysis and enrichment analysis on the pathways of DMs. The metabolic pathways were further screened to find the key metabolic pathways with the highest correlation with metabolites.

Results

Clinical information

The clinical parameters, including age, parity, body condition score (BCS), and milk yield, are shown in Table 1. The differences among the two groups in number of dairy cows, age, parity and BCS were not significant ($P > 0.05$). However, cows with IO have higher daily milk yield ($P < 0.05$).

Table 1
Clinical information in two groups of the tested cows

Project	Estrus cows (n = 6)	Inactive ovary cows (n = 6)
Age	3.37 ± 0.54	3.13 ± 0.92
Parity	2.33 ± 0.52	2.00 ± 0.89
BCS	2.92 ± 0.49	2.67 ± 0.20
Milk yield	38.48 ± 3.47	44.00 ± 4.17*
Note: "*" Means significant difference. BCS = body condition score		

Follicle development information

Table 2 shows the follicular development information of 12 dairy cows 50-55d postpartum. The diameter of the dominant follicles of IO group are significantly smaller than E group ($P < 0.01$). The growth rate of dairy cows in the IO group was extremely slower than that in the E group ($P < 0.01$).

Table 2
Follicle development at 50-55d postpartum

follicle diameter	Estrus cows (n = 6)	Inactive ovary cows (n = 6)
50 d postpartum (mm)	6.67 ± 1.70	5.33 ± 0.56
55 d postpartum (mm)	13.67 ± 0.71	7.33 ± 0.42**
Growth rate (mm/d)	1.40 ± 0.21	0.40 ± 0.17**
Note: "**" Means significant difference.		

Serum Biochemical Indicator Levels

Table 3 shows the serum biochemical indexes of the two groups of cows. Compared with the E group, GLU concentration was decreased ($P < 0.05$), BHBA and NEFA concentrations were increased ($P < 0.05$) in the IO group and there was no difference in other indicators between the two groups.

Table 3
Serum biochemical parameters level in two groups of the tested cows

Project	Estrus cows (n = 6)	Inactive Ovary cows (n = 6)
BHBA (mmol/L)	0.78 ± 0.37	1.37 ± 0.49*
NEFA (mmol/L)	0.44 ± 0.09	0.73 ± 0.25*
GLU (mmol/L)	3.63 ± 0.35	2.99 ± 0.20*
Ca (mmol/L)	2.07 ± 0.28	2.12 ± 0.23
P (mmol/L)	1.85 ± 0.32	1.63 ± 0.22
Mg (mmol/L)	1.22 ± 0.11	1.20 ± 0.09
ALT(U/L)	16.83 ± 7.78	10.31 ± 1.21
AST(U/L)	52.67 ± 21.59	41.00 ± 9.70
TP (g/L)	55.05 ± 17.71	44.07 ± 9.41
Note: “*” Means significant difference. BHBA = β -hydroxybutyrate; NEFA = non-esterified fatty acid; GLU = glucose; Ca = calcium; P = phosphorus; Mg = magnesium; ALT = alanine aminotransferase; AST = aspartate aminotransferase; TP = total protein.		

Analysis of Serum and FF Metabolomics

Orthogonal partial least squares discriminant analysis (OPLS-DA) and permutation test

The scatter plots and permutation tests of OPLS-DA models in the E group and the IO group are shown in Fig. 1. The results of OPLS-DA score chart showed that the two groups of serum and FF samples in the positive and negative ion modes were clearly distinguished and both were in the confidence interval of 95%. The R²_Y of serum positive ion mode, negative ion mode, FF positive ion mode and negative ion mode were 0.68, 0.9, 0.81 and 0.77, which were all close to 1. This showed that the constructed models were in line with the true sample data and the intercept of the Q² regression line and the vertical axis of the positive and negative ion model were less than zero. When the replacement retention decreased, the proportion of Y variable gradually increased and Q² gradually decreased. This showed that the built model had good robustness and no over-fitting phenomenon.

DMs Screening

Table 4 shows the different metabolites in the serum and FF of the two groups of experimental cows. Serum of cows in the IO group had increased in L-glutamine, guanidinoacetic acid, DL-citrulline, and lactose ceramide (d18:1/16:0), L-arginine, phosphatidylcholine, glutamate, sphingomyelin(d18:1/18:1(9Z)), (R)-3-hydroxybutyric acid and alpha-ketoisovaleric acid, and decreased

in L-ribulose, D-xylose, 4-pyruvate and indoleacetic acid. FF of cows in the IO group had increased in choline, L-glutamine, lysophosphatidylcholine (14:1(9Z)), phosphatidylcholine (22:4(7Z,10Z,13Z,16Z)/14:0), phosphatidyl ethanolamine (18:4(6Z,9Z,12Z,15Z)/P-18:1(11Z)) and urocanic acid, arachidonic acid and D-maltos and decreased in 6-Hydroxy-5-methoxyindole glucuronide, ketoleucine, L-valine, phenylpyruvic acid, gentisic acid and 11,12-DiHETrE. A total of 28 different serum and FF metabolites were screened in the two models, of which 18 were increased. and 10 were decreased.

Table 4
Differential metabolites in the serum and FF of IO and E groups

Category	ID	Metabolites	RT (min)	VIP ^a	P-value ^b	FD ^c	mode ^d
Serum	1	L-Glutamine	393.81	1.48	0.0222	↑	ESI+
	2	Guanidoacetic acid	367.36	1.31	0.0153	↑	ESI+
	3	Citrulline	429.12	1.63	0.0060	↑	ESI+
	4	L-Arginine	548.22	1.40	0.0411	↑	ESI+
	5	L-Glutamic acid	315.66	1.42	0.0216	↑	ESI+
	6	4-Pyridoxic acid	34.89	1.73	0.0492	↓	ESI-
	7	Indoleacetic acid	149.56	2.47	0.0404	↓	ESI-
	8	Alpha-ketoisovaleric acid	64.89	1.83	0.0299	↑	ESI-
	9	(R)-3-Hydroxybutyric acid	247.68	2.10	0.0074	↑	ESI-
	10	Lactosylceramide (d18:1/16:0)	207.64	1.41	0.0178	↑	ESI+
	11	PC (16:1(9Z)/16:0)	201.46	1.68	0.0112	↑	ESI+
	12	SM(d18:1/18:1(9Z))	200.91	1.34	0.0387	↑	ESI+
	13	D-Xylose	154.16	2.00	0.0252	↓	ESI-
	14	L-Ribulose	315.92	2.52	0.0085	↓	ESI-
FF	1	L-Valine	302.69	1.92	0.0001	↓	ESI-
	2	L-Glutamine	406.31	1.47	0.0337	↑	ESI+
	3	Ketoleucine	57.88	1.83	0.0018	↓	ESI-
	4	Phenylpyruvic acid	92.35	1.41	0.0330	↓	ESI-
	5	Gentisic acid	61.34	1.44	0.0233	↓	ESI-
	6	Choline	280.86	1.70	0.0100	↑	ESI+
	7	LysoPC (14:1(9Z))	211.14	1.85	0.0069	↑	ESI+
	8	PC (22:4(7Z,10Z,13Z,16Z)/14:0)	36.10	1.13	0.0403	↑	ESI+

Note: IO: Inactive ovaries; E: Estrus; number, ID: Identity; RT: retention time; VIP: Variable Importance for the Projection; FD: Find the difference; ESI: Electrospray ionization; FF: Follicular fluid; PC: Phosphatidylcholine; SM: Sphingomyelin; PE: Phosphatidyl ethanolamine; 11,12-DiHETrE: (5Z,8Z,14Z)-11,12-Dihydroxyeicosa-5,8,14-trienoic acid. "a" is the VIP in the OPLS-DA model (VIP > 1); "b" is the P value obtained by t-test (P < 0.05); "c" is the condition of serum metabolites in cows with IO, where "↓" indicates that the content of the IO group decreased; "↑" indicates that the content of the IO group increased.

Category	ID	Metabolites	RT (min)	VIP ^a	P-value ^b	FD ^c	mode ^d
	9	PE (18:4 (6Z,9Z,12Z,15Z) /P-18:1(11Z))	58.74	1.68	0.0153	↑	ESI+
	10	Arachidonic acid	46.47	1.45	0.0435	↑	ESI-
	11	11,12-DiHETrE	78.28	1.66	0.0098	↓	ESI-
	12	D-Maltose	416.44	1.33	0.0276	↑	ESI-
	13	6-Hydroxy-5-methoxyindole glucuronide	313.81	1.59	0.0043	↓	ESI+
	14	Urocanic acid	322.52	1.73	0.0202	↑	ESI+
Note: IO: Inactive ovaries; E: Estrus; number, ID: Identity; RT: retention time; VIP: Variable Importance for the Projection; FD: Find the difference; ESI: Electrospray ionization; FF: Follicular fluid; PC: Phosphatidylcholine; SM: Sphingomyelin; PE: Phosphatidyl ethanolamine; 11,12-DiHETrE: (5Z,8Z,14Z)-11,12-Dihydroxyeicosa-5,8,14-trienoic acid. "a" is the VIP in the OPLS-DA model (VIP > 1); "b" is the P value obtained by t-test (P < 0.05); "c" is the condition of serum metabolites in cows with IO, where "↓" indicates that the content of the IO group decreased; "↑" indicates that the content of the IO group increased.							

Pathway analysis

Using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, the different metabolites in the pathway between IO cows and E cows were identified and represented by bubble diagram. The pathway analysis is shown in Fig. 2.

The common different metabolites of the serum and FF were L-glutamine and phosphatidylcholine and the levels were increased, while the other metabolites were all different. In amino acid metabolism, D-glutamine and D-glutamate metabolism, alanine, aspartic and glutamate metabolism, arginine and proline metabolism, valine, leucine and isoleucine biosynthesis and phenylalanine metabolism were the main enrichment pathways. In lipid metabolism, glycerophospholipid metabolism and arachidonic acid metabolism were the main enrichment pathways. In carbohydrate metabolism, pentose and glucuronate interconversions and starch and sucrose metabolism were the main enrichment pathways and were all closely related to the occurrence of diseases.

Discussion

Follicle stimulating hormone increased after cows parturition (lasting 2 ~ 3 d). When the follicle grows to a certain stage, the difference between the growth rate of the largest follicle and the second largest follicle reaches the maximum, and follicle deviation occurs (Ginther. 2016). The maximum diameter of the follicle may decrease due to the decrease of luteinizing hormone (LH) pulse. When the progesterone (P₄) level in the blood decreases, the number of LH pulses will increase, and the diameter of the dominant follicle will continue to grow (Cooperative Regional Research Project. 1996). However, the dominant

follicles fail to ovulate due to inadequate LH production, which is the direct cause of IO. In addition, high-producing cows with subclinical ketosis had low cholesterol or high liver perfusion since high energy metabolic demand, which may reduce steroid hormones synthesis or increases the clearance rate of ovarian steroid hormones, and then leads to anovulation and persistent corpus luteum (Petersson et al. 2006). The consumption of excessive lactation in high-yielding dairy cows were unable to provide more energy for the reproductive system, it may hinder the growth and development of follicles (Butler et al. 1981).

In our study, the clinical estrus, follicular development, uterine involution, ovarian stiffness and dominant follicle diameter were examined at 50 and 55 days postpartum. Our results showed that cows with IO had an average less than 8mm diameter of follicles (7.33 ± 0.42 mm) and less than 3mm/d growth rate (0.4 ± 0.17 mm/d) within 5 days, and the other cows showed normally estrus behaviors and dominant follicles diameter (13.67 ± 0.71 mm), growth rate (1.4 ± 0.21 mm/d). Therefore, the follicular fluid of IO cows are collected in the secondary follicle stage (Nelson et al. 2017).

By comparing the serum biochemical indicators of E cows and IO cows in this study, the serum BHBA and NEFA levels of IO cows are higher, while the glucose is lower. It suggests an obvious subclinical ketosis (SCK) feature (Newman et al. 2016; Ihsanullah et al. 2017). Studies by Chang et al (2019) have also confirmed that after cows developed SCK postpartum 14–21 days, nearly 50% of them showed IO during postpartum 60–90 days. Serum BHBA and NEFA can inhibit the survival and growth of sheep preantral follicles and their oocytes cultured in vitro (Nandi et al. 2017). Glucose, TC and BHBA affect the oocytes maturation ability in dairy cows (Ferreira et al. 2011).

Changes of Serum Metabolites in IO Cows

Amino acids are one of the important components of protein and milk fat. In some early reports, some amino acids may affect the milk quality and production performance of dairy cows (Giallongo et al. 2016; Lee et al. 2015). In this study, there were seven serum DMs directly involved in amino acid metabolism in IO cows. Previous studies have confirmed that glutamine and glutamate can be converted into each other and directly participate in the tricarboxylic cycle for gluconeogenesis (Ardawi & Newsholme, 1990). Guanidinoacetic acid can generate creatine (Ostojic, 2019), in the form of phosphocreatine and free creatine, which provides energy for the body, alpha-ketoisovaleric acid can be converted into valine and together with isoleucine, it can regulate the blood sugar level to provide energy for the body (Xu et al. 2016). This study found that urea circulation will also be affected by participating in the tricarboxylic cycle since affecting the formation of fumaric acid through amino acids such as arginine (Yoshimi et al. 2016), or involving in the synthesis of antioxidants and anti-inflammatory factors (Liang et al. 2018; Zhao et al. 2018). The specific mechanism of amino acid metabolism affects inactive ovaries through energy metabolism or inflammation were still unclear until now.

The carbohydrates screened in this experiment mainly involve pentose and glucuronate interconversions. For providing energy, the pentose phosphate pathway can offer raw materials for the synthesis of other substances, such as nucleotides and amino acids (Luo et al. 2007). In addition, L-ribulose can generate

ribitol and carry out riboflavin metabolism, which participates in the energy response of the respiratory chain and cell growth and metabolism (Pinto & Zemleni, 2016). The blood L-ribulose and D-xylose levels are therefore reduced to maintain the level of pyruvate and some metabolic pathways such as the pentose phosphate pathway and riboflavin metabolism are weakened, which is not conducive to follicular development.

The study by Luo et al. (2019) showed that the content of phosphatidyl -choline in the serum of dairy cows increased during early lactation, which is consistent with these experimental results. Phosphatidylcholine homeostasis is particularly important for maintaining cell survival and growth. Studies have found that the total amount of phosphatidylcholine in cells is related to cell growth and apoptosis, positively related to growth and negatively related to apoptosis (Leng et al. 2018). Under the action of phospholipase, phosphatidylcholine can increase the production of arachidonic acid and linoleic acid (Seeley et al. 2006). The 13-HpODE produced by the oxidative metabolism of linoleic acid can enhance epidermal growth factor signal transduction by participating in the dephosphorylation of epidermal growth factor receptors (Hui et al. 1999) and inducing the expansion of the cumulus in the ovaries of mammals. Sphingomyelin can be metabolized to produce ceramide, which has important functions in barrier function, regulating cell function and participating in the signal transduction process which regulates cell growth and apoptosis (Hage et al. 2014). When culturing cells in vitro, adding ceramide can cause protein kinase inactivation and reduce the absorption of glucose by cells, and accelerate cell apoptosis (Hage et al. 2014). However, the relationship between ceramide and IO in dairy cows needs further study. In addition, this study found that 4-pyridoxine, a metabolite of vitamin B6, decreased in the serum of cows with IO. 4-Pyridoxic acid was formed from pyridoxal by aldehyde oxidase (AOX) in the liver. In previous studies among diabetic patients with vascular risk, 4-pyridoxine, and PAR [ratio of 4-pyridoxic acid/(pyridoxal + pyridoxal 5'-phosphate)] index was higher in plasma and urine (Obeid et al. 2019), but its relationship with IO needs to be further explored.

Changes of FF Metabolites in IO Cows

In this study, the change trend of glutamine in FF and serum was consistent. The L-glutamine is the precursor and main energy source of nucleic acid biosynthesis, which enters the glycolysis and gluconeogenesis pathways or the purine and pyrimidine metabolism pathway. It can provide energy for the body (Summers et al. 2005), and promote the proliferation of cumulus cells in vitro (Sutton-McDowall et al. 2010). Excessive glutamine is transported to the cell through the transport system alanine-serine-cysteine (ASC), which may act as a competitive inhibitor of cysteine uptake. Cysteine is a key factor in the synthesis of glutathione in the g-glutamyl cycle (Yin et al. 2016), but it is still unclear whether glutamine in dairy cows with IO has an effect on the synthesis of glutathione, which in turn oxidizes and damages the follicular cells and hinders the development of the follicle. Other metabolites, such as L-valine, phenylpyruvic acid, and gentisic acid, can all participate in the tricarboxylic cycle, which in turn affects carbohydrates metabolism and lipid metabolism (Grochowska et al. 2001; Lemmon & Schlessinger, 2010).

The carbohydrates selected in FF are mainly related to starch and sucrose metabolism, pentose and glucuronate interconversions, and D-maltose as an intermediate substance that can convert glycogen into glucose. During the maturation of oocytes, both glucose and 6-hydroxy-5-methoxyindole glucuronide can provide energy for cells. In addition, glucose can also synthesize extracellular matrix substrates by cumulus expansion and O-linked glycosylation in cell signal transduction through the hexosamine biosynthesis pathway for follicular growth and regulate oocyte nuclear maturation and redox state through the pentose phosphate pathway (Cetica et al. 2002). Furthermore, 6-Hydroxy-5-methoxyindole glucuronide generates ascorbic acid which resists oxidation and scavenges free radicals (Chatterjee, 1978). In this study, the increase in maltose in the FF of IO cows and the decrease in 6-Hydroxy-5-methoxyindole glucuronide may enhance glycogenolysis.

Lipid-related metabolites mainly involve in glycerophospholipid metabolism and arachidonic acid metabolism. Phosphatidylinositol can be produced from serine and then enter the tricarboxylic cycle through acetyl-CoA or indirectly produce choline. It can also participate in the glycosylphosphatidylinositol (GPI) metabolic pathway, allowing cell membranes to bind to proteins (Jope et al. 1979; de Almeida et al. 2003). Studies have shown that choline can generate acetylcholine and enter the cAMP signaling pathway. Acetylcholine is a neurotransmitter, which not only affects the permeability of the membrane to ions, but also transmits signals through some second messengers and affects the physiological metabolic process. Studies have shown that in the ovaries cAMP is instrumental as a second messenger for the follicle stimulating hormone (FSH) and luteinizing hormone (LH) receptors (Conti, 2002; Zhang et al. 2004; Menon & Menon, 2012). The increased metabolism of glycerophospholipids may be related to signal transduction during follicular development of IO cows with SCK, but further research is needed. Arachidonic acid is elevated, which is consistent with the results of Moore S G (Wathes et al. 2007). Arachidonic acid participates in the ovarian production of steroid hormones through its metabolites, such as cyclooxygenase metabolism, to produce PGE2 and PGF2 α , and lipoxygenase metabolism to produce leukotrienes. The PGE2 can promote the synthesis of hyaluronic acid and the expansion of the cumulus, or 5-HPETE produced by metabolism, which can affect the production of steroid hormones by regulating the expression of StAR protein (Eppig, 1981; Wang et al. 2003). However, Zhang (2019) pointed out that high concentrations of arachidonic acid can induce the death of granulosa cells in the ovary and inhibit the synthesis of estrogen by granulosa cells. This result may be because the catabolism of arachidonic acid is decreased and the content of FF is increased, which affects the synthesis of steroid hormones in granular cells.

Comparison of Metabolites in Serum and FF of IO Cows

In this experiment, UHPLC-QTOF-MS was used to analyze the serum and FF samples of the cows in the E and IO cows and 28 different metabolites were obtained. According to KEGG analysis of metabolic pathways, an interaction correlation diagram between DMs was constructed through integration in Fig. 3.

Scaramuzzi et al. (2011) pointed out that the SCK affects the growth of follicles induced at different levels of the hypothalamic-pituitary-ovarian axis (Scaramuzzi et al. 2011). Rodgers & Irving-Rodgers

(2010) reviewed the regulation of follicular growth, fertility, and oocyte quality in ruminants. The FF from the blood needs to pass through the cortical interstitium, the basal layer of the follicle and the granular cell layer of the wall (Senbon et al. 2003). As the follicle develops, fluid will accumulate in the antral cavity of the follicle to provide nutrients for the development and maturation of the oocyte. The main function of follicles is to provide a blood to follicular barrier and create a favorable environment for growing oocytes. Similarly, oocytes play an important role in promoting the growth of follicles and directing the differentiation of granulosa cells, and interact with surrounding somatic cells such as granulosa cells (Senbon et al. 2003). In this study, L-glutamine and L-glutamate in the FF and serum were increased in dairy cows with IO, which was not conducive to improve follicular development. The increased L-glutamine in FF should derive from L-glutamate in the blood and then converted to pyruvate, which can provide energy for follicular development (Ardawi & Newsholme, 1990), mainly via alanine, aspartate and glutamate metabolism. In IO cows, the serum and FF phosphatidylcholine and its upstream and downstream metabolites were all elevated, mainly due to arachidonic acid metabolism. Although an appropriate concentration of arachidonic acid can participate in the production of steroid hormones, the high content of arachidonic acid in FF can induce granular cell death, which is not conducive to follicular development (Eppig, 1981; Wang et al. 2003). Thus, this study showed that sphingomyelin and lactosylceramide may be related to IO in dairy cows, but the specific mechanism needs further confirmation in the future.

Conclusion

UHPLC-QTOF-MS metabolomics was used to analyze the DMs of IO cow serum and FF and performed integrated analysis. The main pathways involved were D-glutamine and D-glutamate metabolism, alanine, aspartic and glutamate metabolism, arginine and proline metabolism, valine, leucine and isoleucine biosynthesis, phenylalanine metabolism, glycerophospholipid metabolism, arachidonic acid metabolism, pentose and glucuronate interconversions and starch and sucrose metabolism. Among them, glycerophospholipid metabolism mainly involved signal transduction during follicular development, which requires further study. Among the selected DMs, we found sphingomyelin and lactosylceramide may be related to IO in dairy cows, but it is still unclear how the specific DMs affect IO development and needs further in-depth study by expanding the number of cows and other research in the future.

Declarations

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Author contributions

Zhijie Wang and Yuxi Song conceived, drafted, and wrote the manuscript. Shuhan Sun prepared the tables and figures. Chang Zhao and Feng Zhang conducted related experiments. Yunlong Bai and Yingying Zhang provided the samples and materials. Cheng Xia and Shixin Fu critically reviewed the manuscript. All authors approved the final version of the manuscript.

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Figures

Figure 1

OPLS-DA model analysis of E group versus IO group in positive and negative ion mode. Note: A and B represent serum and follicular fluid samples. The prediction score of the first principal component of the scatter plot is the abscissa, the orthogonal principal component score is the ordinate, red is the E group

and blue is the IO group. In the OPLS-DA permutation plot, the permutation retention rate is the abscissa, the value of R2Y or Q2 is the ordinate, the blue dot is the Q2 value, the green dot is the R2Y value, and the dotted line is the regression line.

Figure 2

Bubble diagrams of serum and follicular fluid samples Note: The position and size of the bubble on the abscissa is the size of the influence factor of the path in the topological analysis. The larger the bubble,

the greater the influence factor; the position and color of the bubble on the ordinate is the P value in the enrichment analysis (-ln P-value, i.e. negative Natural logarithm), the smaller the P value, the darker the color, and the more significant the enrichment.

Figure 3

Differential metabolites network metabolism map Note: ↑ and ↓ represent increase and decrease. Blue means follicular fluid samples; green means serum samples; and red means both.