Polish Journal of Veterinary Sciences Vol. 23, No. 1 (2020), 59–67

DOI 10.24425/pjvs.2020.132749

Original article

Plasma metabolic differences in cows affected by inactive ovaries or normal ovarian function post partum

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Abstract

Anestrus is essential to an unsuccessful pregnancy in dairy cows. One of the many factors that influences anestrus is the inactive ovary. To characterize in detail the plasma metabolic profile, anestrus cows suffering from inactive ovaries were compared with those with natural estrus. The Holstein cows 60 to 90 day postpartum in an intensive dairy farm were assigned into inactive ovaries groups (IO, n=20) and natural estrus group (CON, n=22) according to estrus signs and rectal palpation of ovaries. Plasma samples from two groups of cows were collected from the tail vein to screen differential metabolites using gas chromatography/mass spectrometry (GC/MS) techniques and multivariate statistical analysis and pathways. The results showed that 106 compounds were screened by GC/MS and 14 compounds in the IO group were decreased by analyzing important variables in the projection values and *p* values of MSA.Through pathway analysis, 14 compounds, mainly associated with carbohydrate metabolism and amino acid metabolism, were identified to results in IO, which may seriously affect follicular growth. Metabolomics profiling, together with MSA and pathway analysis, showed that follicular growth and development in dairy cows is related to carbohydrate and amino acid metabolism by a single or multiple pathway(s).

Key words: dairy cows, inactive ovaries, gas chromatography/mass spectrometry, multivariate statistical analysis, differential metabolites

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Introduction

The reproductive performance is an important index to assess productivity of dairy cows. The process after calving, from follicular emergence, deviation, growth and ovulation to fertilization and pregnancy, may culminate in infertility if any step is abnormal. When the follicle diameter is less than 4 mm, and follicle deviation does not occur, this state is defined as "inactive" or "smooth" (Peter et al. 2009). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are one of the key factors affecting follicular development. However, the metabolic disorder is a secondary factor leading to inactive ovaries (IO). Many metabolites and hormones can directly affect the growth of follicles, including growth hormone, insulin-like growth factor-1, insulin, alanine, glutamine, etc. (Zhang J et al. 2018), which are associated with the process of follicular growth (Comin et al. 2002, Fair 2010). Many studies have explored the above problems. Rieger (1994) reported that the level of pyruvic acid metabolized by the oocyte doubly when the follicle begins to grow. It will return to a normal level 24 hours after the follicle maturation. Other research suggests that, before transport to the oocyte, pyruvic acid is synthetized from glucose and lactic acid in cumulus cell (Leese and Barton. 1985). Nandi et al. (2008) demonstrated that different concentrations of glucose, lactate and pyruvate had an effect on cultured oocytes, and granulosa and cumulus cells of buffalo and sheep. Although many metabolites have been shown to be related to follicular growth, the details remain unknown.

In dairy cows, follicular waves are characterized by the periodicity of follicular development (Ginther. et al. 2000, Ginther. et al. 2001). The first follicular wave arise approximately on the 4th day after calving, and the follicles continue to grow during estrus, which lasts for 21 days. When mature follicles appear on the surface of the ovary, cows show mounting behavior. In general, dairy cows do not show esturs behavior during the 1st and 2nd follicle wave after calving because their uterus and body condition need to return to normal. It is well known that cows are difficult to conceive during the first two estrus postpartum, and 60 to 90 days postpartum during the three to four esturs is the appropriate time for conception.

It will be a new breakthrough or strategy to reveal inactive ovaries in dairy cows using metabolomics technology. Muñoz et al. (2013) reported that the sex of the embryo may be identified using metabolomics combined with bioinformatics. Moreover, metabolomics has been carried out to assess the outcome of encyesis (Singh and Sinclair, 2007). Gas chromatography and mass spectrometry (GC/MS) are combined to improve the sensitivity of detection of low abundance metabolites (Luo et al. 2019) when compared with nuclear magnetic resonance (Dettmer et al. 2006). Bender et al. (2010) used GC/MS to analyze the follicular fluid of primiparous and multiparous dairy cows. The research suggested that the contents of the follicular fluid in multiparous cows have an unfavorable effect on follicular growth, compared with those of primiparous animals.

In a recent metabolomics study, Xu et al. (2016) performed plasma metabolic profiling of dairy cows with ovarian inactivity using only nuclear magnetic resonance. They noted that the metabolism of glucose, amino acids, lipids and choline were disordered in relation to follicular growth. Therefor, the aim of this study was to screen for unknown metabolic disorders related to follicular growth using GC/MS.

Materials and Methods

Animal selection

All animals involved in this study were cared for in accordance with the principles of the Heilongjiang Bayi Agricultural University (Number: 20160320-1). The study was carried out in an intensive dairy farm of Heilongjiang Province, China, The cows were fed in free-tie bar and a total mixed ration diet during early lactation, which consisted of 8.5 kg of concentrate, 18 kg of silage, 3.5 kg of hay, and 300 g fat. Their nutritional level on a DM basis included 55.50% DM, 16.10% crude protein, 7.33 MJ·kg-1 net lactation production, 5.40% fat, 39.20% NDF, 20.20% ADF, 175 g of calcium, and 110 g of phosphorus. The basal diet was formulated to meet the nutrient requirements according to the Feeding Standards of Dairy Cattle in China. All tested cows with similar age, parities, and milk yield were in turn assigned into the inactive ovaries group (IO) and natural estrus group (NE). The selection criteria of IO and NE were as follows and all information of the tested cows were listed in Table 1.

Dairy cows selected for the IO group: 20 cows at 60 to 90 days after calving were selected as IO animals if they showed no mounting behavior, and there were no follicles or its diameter was than 4mm on the surface of the ovaries according to B ultrasound imaging, which lasts 7 days.

Dairy cows selected for the natural estrus group (NE): Because hormonal and follicular conditions vary on different days during estrus, 22 cows at the similar days after calving as the IO samples were selected as NE animals if they showed mounting behavior, and there were growing follicles or its diameter more than 8 mm on the surface of the ovaries according to B ultrasound imaging, which lasts 7 days.

Plasma samples

The 10 ml blood of all tested cows were collected from tail vein before fed in the morning, in tubes with anticoagulant. After centrifugation at 1500×g for 5 min, the supernatant collected was centrifuged at 12,000 g for 5 min, and frozen at -80°C.

Hormone detection

Progesterone (P4) (SBJ-B0063), LH(SBJ-B0064), FSH(SBJ-B0061) and E_2 (estradiol) (SBJ-B0062) concentrations were assayed using ELISA kits from Senbeijia, Nanjing, China.

Sample preparation

50 μL plasma sample thawed was added into 10 μL L-2-chloro- phenylalanine (internal standard), and were vortexed for 10s. 150 μL of methanol and acetonitrile (2:1) mixed solution were added to precipitate protein in the sample, and was vortexed for 1 min. After standing at 20°C for 10 min, the sample was ultrasonically extracted for 10 min at low temperature, and then left standing for 10 min at 20°C again. Subsequently, the sample was centrifuged (15000 × g, 4°C), and the 150 μL supernatant was extracted into a glass bottle. After this, 80 μL methoxyamine hydrochloride (15 mg/mL) was added, and the sample was vortexed for 2 min. The sample was placed in a 37°C shaking incubator for 90 min, sample were added with 80 μL of the derivatives of BSTFA with 1% trimethylchlorosilane and 20 μL hexane, and the sample was vortexed for 2 min. After 60 min reaction in 70°C incubator, the sample was left to stand for 30 min, following which the sample was analyzed by GC/MS.

The quality control (QC) sample was done by mixing aliquots of all samples to form a pooled sample, and then analyzed by the same method. The QCs were injected at every five samples throughout analysis. The aim is to provide a set of data for evaluating the test and instrument by the repeatability of QCs.

Gas chromatography–mass spectrometry analysis

Agilent Analyzed instrument for GC-TOF-MS (Gas Chromatography/Time of Flight/Mass Spectrometry, 7890A-5975C). Was used of the derivatized solution was added into each split mode. The GC-TOF-MS temperature program began at 50°C, followed by 15°C per min oven temperature ramps to 125°C, 5°C per min to 210°C, 10°C/min to 270°C, and 20°C /min to 305°C, and a final 5 min maintenance at 305°C. The electron impact ion source was held at 230°C with a filament bias of –70 V. Full scan mode (m/z 50−600) was used, with an acquisition rate of 20 spectra/second in the MS setting.

Data preprocessing and multivariate statistical analysis

Data preprocessing

The raw data were preprocessed using ChromaTOF software (V4.34, LECO). The data included information on the sample, the time of flight, the ratio of charge/ /mass, and the peak intensity. Normalization was the main method used to reprocess the raw data.

Multivariate statistical analysis

All experimental results were analyzed by SIMCA-P + 14.0 (Umetrics). The results were subjected to principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Metabolite changes were analyzed using principal component analysis (PCA) and orthogonal partial least squares discrimination (OPLS-DA).

In this study, the default seven-round cross-validation was applied with one-seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting.

Identification of differential metabolites

All differential metabolites between the two groups were identified using the MSA method and Wilcoxon−–Mann−Whitney test. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1.0 are considered relevant for group discrimination. Metabolites with both multivariate and univariate statistical significance (VIP > 1.0 and $p < 0.05$) were annotated with the aid of available reference standards in our laboratory, and using the NIST 11 standard mass spectral databases and the Feinh databases linked to Chroma TOF software. Similarity of more than 70% can be considered as reference standards.

Pathway analysis

The pathway analysis was performed using the KEGG database. All differential metabolites were searched by KEGG. The pathways related to follicular growth were filtered to explore the potential relationships.

Table 1. Information and hormone data from dairy cows with IO and NE.

Variable mean (± SD)†	IO (N=20)	NE (N=22)	*p*-value ‡
Follicular diameter (mm) §	2.25 (1.37)	10.82 (2.30)	0.00**
DM for blood collection sample ¶	72.90 (8.32)	73.82 (9.06)	0.74
DM for ovulation ¶	NA ††	79.59 (8.91)	NA††
Age (years)	3.06 (1.21)	3.19 (1.35)	0.76
Parity	1.75 (0.91)	1.19 (1.06)	0.61
BCS	2.66 (0.25)	2.80 (0.18)	0.05
Milk yield (kg/day)	34.16 (12.53)	33.04 (7.11)	0.72
Milk yield (kg/year)	3915.35 (1251.75)	3969.82 (1115.84)	0.88
FSH (mIU/mL)	9.80 (3.83)	8.83 (3.94)	0.42
LH (ng/L)	30.76 (11.09)	27.96 (15.27)	0.50
E_2 (pg/mL)	85.64 (24.16)	141.43 (24.16)	0.00**
P4 (ng/mL)	0.12 (0.05)	0.10 (0.05)	0.17

† SD standard deviation, N number of experimental animal samples BCS body condition score, FSH follicle-stimulating hormone, LH luteinizing hormone, E_2 estradiol, P4 Progesterone, IO inactive ovaries, NE natural estrus.
‡ p-values have been calculated using the t-test; measures with very significant differences (p value<0.01) have been marked with **.
§ The follicular diameter is shown to the largest follicle on surface of ovary.
¶ DM days of milking for blood collection samples.
†† "NA" means no data, because the dairy cows with IO can't ovulate without treatment.

Results

Information and hormone levels of the tested cows

Table 1 shows the information and hormone levels in two groups of cows. Compared with the NE cows, there was no significant difference in the age, parity, BCS, milk yield (day and year), FSH, LH, P4 ($p>0.05$) in the IO cows. However, the IO cows had significantly lower E_2 levels ($p<0.01$) than that of the NE cows.

Validation of GC/MS method performance

Figure 1 is a typical total ion current (TIC) of plasma samples from the IO group and the NE group. In Fig. 1, the analytical performance of the GC/MS method was evaluated using QC samples. It showed that the TIC of the QC samples overlapped and the reproducibility of retention time and relative abundance was good.

Multivariate statistical analysis

Principal components analysis

With the dataset collected, principal components analysis (PCA) was used to provide an integrated view of the differences in metabolome organization between the two groups. The unsupervised method clearly discriminated the samples, with PC1 and PC2 explaining 33.7% and 7.21% of the total variance, respectively. As can be seen on the PCA score plot, the pronounced separation based component 1 (t1) was obtained for the IO group, which differed strongly from the NE group in Fig. 2.

Orthogonal partial least squares discriminant analysis

Orthogonal partial least squares discriminant analysis (OPLS-DA) is the most appropriate technique to search for metabolic profiles because the predictive component of OPLS-DA can describe a treatment effect while excluding the variance between samples in the same group showed in Fig. 3a. OPLS-DA model uses R^2 and Q^2 value to detect models without over-fitting and predictive function. R^2 refers to the parameter of goodness of fit, and Q^2 refers to the parameter of predictive ability. When the R^2 and Q^2 values are both greater than 0.5, the established OPLS-DA model is a high quality model. In Fig. 3b, the values of R^2Y and Q^2 were greater than 0.5 ($R^2Y=0.983$, $Q^2=0.96$), and the R^2/Q^2 was close to 1 (ratio≈1.0232). The results show that the OPLS-DA model of the experiment is suitable and predictive.

Differences in metabolites between the two groups

106 compounds were identified by GC/MS, and 14 metabolites were found via the VIP value

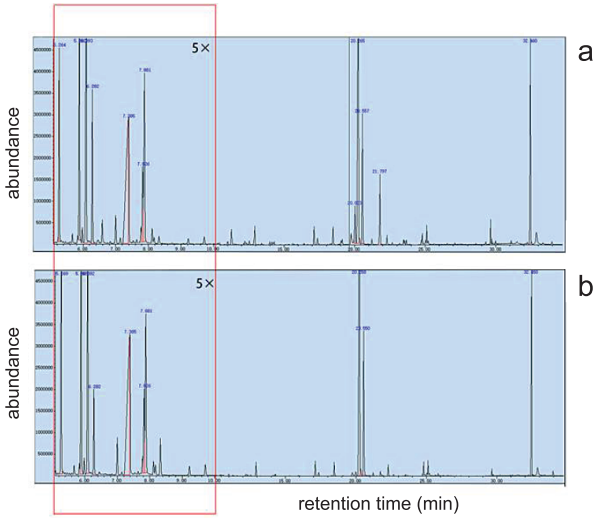

Fig. 1. Total ion currents of plasma samples from natural estrus (a) and inactive ovaries (b) group samples by GC/MS. The phase from 0–10 min is magnified five times compared with the other stages.

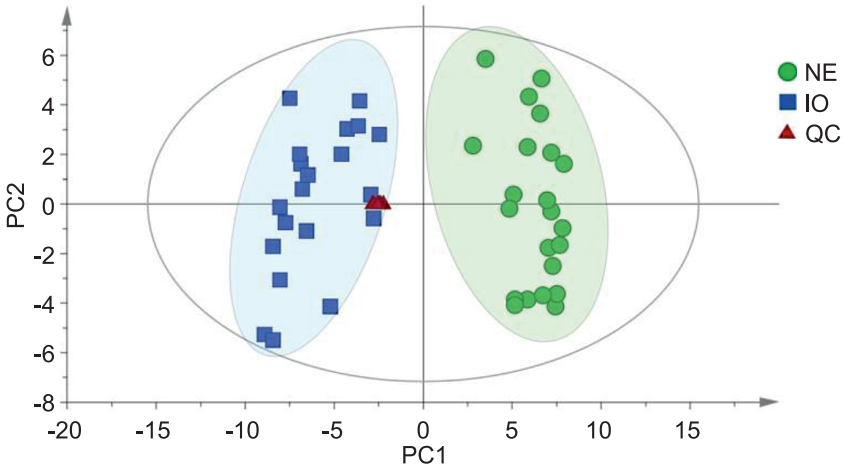

Fig. 2. Principal components analysis (PCA) scores plot of dairy cow plasma GC/MS with inactive ovaries (squares and blue), NE (circles and green) and QC samples (triangles and red).
Sample clustering is observed due to principal component 1 (PC1=33.7%) and principal component 2 (PC2=7.21%).
Abbreviations: NE: Natural Estrus group; IO: Inactive Ovaries group; QC: Quality Control; PC1: Principal Component 1; PC2: Principal Component 2.

and p value (VIP>1, $p<0.05$). In Table 2, the levels of 14 metabolites were lower in the IO group than those of the NE group, including Ribitol, Oxalate, Trehalose, D-tagatose, Hydroxylamine, Vanillylmandelic acid, Phenylpyruvate, Pipecolinic acid, beta-Alanine, 5-Aminoimidazole-4-carboxamide ribotide, 2-Hydroxybutanoic acid, Putrescine, 6-Hydroxy- nicotinic acid and Nicotinoylglycine.

Analysis of metabolic pathways

Metabolic pathway analysis is a tool that explores the biological function of metabolites. In Table 2, 14 differential metabolites were analyzed using the KEGG database, which mainly involved amino acid metabolism, carbohydrate metabolism and energy metabolism. According to analysis of these metabolites' pathways, an integration of diagram in Fig. 4 were build to reveal interaction or relationship of the differential metabolites in the IO cows. These terminal metabolites being at a reduced level suggested that some metabolic pathways were disordered.

Discussion

Table 2 showed that there was a significant difference in plasma metabolites between the IO group and the NE group. These differetial metabolites indicated that the metabolic changes were related to follicular growth. In Fig. 2 and Fig. 3, the PCA and OPLS-DA of plasma metabolites obviously discriminated between

Table 2. Differential plasma metabolites of the studied groups of cows.

Metabolites	KEGG† ID	RT‡ (min)	VIP§	*p*-value¶	IO change††	KEGG Pathway & biological function (KEGG pathway ID)
			Sugars			
Ribitol	C00474	16.35	1.97	1.24E-13	↓	Pentose and glucuronate interconversions (00040)
Oxalate	C00209	23.47	1.85	9.89E-07	↓	Glyoxylate and dicarboxylate metabolism (00630)
Trehalose	C01083	29.29	2.15	2.68E-20	↓	Starch and sucrose metabolism (00500)
D-Tag atose	C00795	19.72	2.17	7.75E-17	↓	Galactose metabolism (00052)
Hydroxylamine	C00192	32.53	1.29	4.19E-05	↓	Nitrogen metabolism (00910)
5-Aminoimidazole--4-carboxamide ribotide	C04677	29.81	1.01	8.88E-04	↓	Biosynthesis of alkaloids derived from histidine and purine (01065)
			Amino acids			
Vanillylmandelic acid	C05584	30.94	1.96	6.94E-10	↓	Tyrosine metabolism (00350)
Phenylpyruvate	C00166	10.15	1.93	9.58E-13	↓	Phenylalanine, tyrosine and tryptophan biosynthesis (00400)
Pipecolinic acid	C00408	7.16	1.59	8.41E-06	↓	Lysine degradation (00310)
2-Hydroxybutanoic acid	C05984	12.32	2.06	1.21E-15	↓	Propanoate metabolism (00640)
beta-Alanine	C00099	5.21	1.51	8.17E-06	↓	Propanoate metabolism (00640) beta-Alanine metabolism (00410)
Putrescine	C00134	27.61	1.17	2.15E-03	↓	Arginine and proline metabolism (00330)
			Nicotinic acid			
6-Hydroxynicotinic acid	C01020	23.02	1.68	4.84E-08	↓	Nicotinate and nicotinamide metabolism (00760)
Nicotinoylglycine	C05380	10.20	1.76	1.52E-09	↓	Nicotinate and nicotinamide metabolism (00760)

† KEGG Kyoto Encyclopedia of Gene and Genomes (KEGG) online database (http://www.kegg.jp).
‡ RT retention time.§ VIP Variable importance in the projection.
¶ p-values have been calculated using the t-test.
†† IO inactive ovaries; "↓" Compared to the natural estrus group, the inactive ovaries cows show down-regulated expression.

the IO group and the NE group. Follicles development need sufficient energy substrates, including carbohydrate, amino acid, and others. In Fig. 4, pathway analysis showed that some metabolic pathways' disorders were involved in the occurrence and development of inactive ovaries.

Effect of carbohydrate metabolism on follicular development

In this study, D-tagatose and trehalose were at a lower level in the IO group. According to the results of pathway analysis, the two substances are related to glucose, and trehalose is an upstream metabolite of glucose in the "Starch and sucrose metabolism" pathway. Granulosa cells play a pivotal role in glucose utilization, having two metabolic pathways for glucose, the "Glycolytic" and "Pentose phosphate pathway" (PPP) (Sutton et al. 2003).

In the glycolytic pathway, pyruvate is a terminal product, which can pass into oocytes. Oocyte growth and development are absolutely dependent on the nurturing capacity of the follicle, in particular of the granulosa cells (Sutton. et al. 2003). Several authors have suggested that glucose and pyruvate are the primary energy source for granulosa cells and oocytes (Biggers 1967, DownsUtecht 1999, Eppig et al. 2000, Cetica et al. 2002). Nandi et al. (2008) reported that glucose, lactate and pyruvate have an effect on cultured oocytes, and on granulosa and cumulus cells. It indicated that glucose and pyruvate are the principal energy sources for oocytes and follicular somatic cells in buffalo and sheep. In particular, pyruvate concentrations appear to be affected by granulosa cell glycolytic metabolism

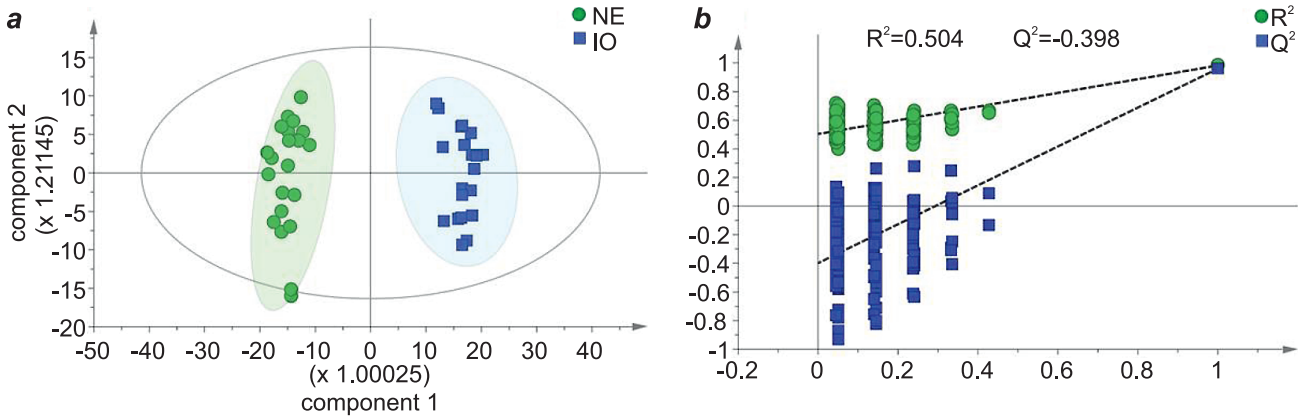

Fig. 3. OPLS-DA results for the NE group (circles and green) and IO group (squares and blue).
a. Score plot between selected groups. It was possible to notice that the groups are different (R2X=0.425, R2Y=0.983, Q2=0.961).
b. Validation plot of OPLS-DA models from the two groups (R2: circles and green Q2: squares and blue).

(Gull et al. 1999). Oocyte maturation conditions that are associated with higher glycolytic pathway activity also promote improving oocyte developmental competence (Krisher and Bavister 1999). In summary, pyruvate concentrations in oocyte cells are positively correlated with the level of glycolytic metabolism.

The PPP process begins with the oxidation of glucose to glucose-6-phosphate and is required for the formation of ribose sugars for DNA and RNA. One of the products of the pathway, phophoribosylpyrophosphate (PRPP) is used by the oocyte for purine synthesis. In Table 2, Ribitol had lower level in the IO group. It may affect the upstream substrate of PRPP, named D-ribulose-5-phosphate, in the PPP and "Pentose and glucuronate interconversions" pathway. In murine oocytes, oxalate and hydroxylamine were involved in "Glyoxylate and dicarboxxylate metabolism" and "Nitrogen metabolism", respectively. Furthermore, these two pathways, "Glyoxylate and dicarboxxylate metabolism" and "Nitrogen metabolism", are subsequent pathway of purine metabolism.

Given the findings related to D-tagatose and trehalose, and to oxalate and hydroxylamine, it was speculated that the carbohydrate metabolism was disordered in dairy cows with IO. All the above findings indicate that the capacity of the oocyte to utilize glucose is positively correlated with subsequent embryo developmental potential (Spindler et al. 2000).

Effect of amino acid metabolism on follicular development

According to the results of GC/MS and pathway analysis, five pathways were found to be related to amino acid metabolism including phenylalanine, tyrosine and tryptophan biosynthesis, tyrosine metabolism, lysine degradation, propanoate metabolism and arginine and proline metabolism. L-tyrosine, pyruvic acid, beta-alanine, arginine and proline had lower levels in the IO group, which are involved in the aboved five pathways.

Amino acids play important roles in the process of follicular development. Sinclair et al.(2008) reported that concentrations of alanine and glycine were the highest in follicular fluid, which were related to cell growth. They are involved not only in protein synthesis but also in osmotic adjustment (Steeves et al. 2003). Matoba et al. (2014) identified the differential metabolites in follicular fluid from those with normal growth and those with abnormal growth using metabolomics. The result showed that follicular growth was positively associated with L-alanine, glycine and L-glutamic acid. Alanine, glycine, proline, valine, L-tyrosine, pyruvic acid, arginine, and proline may pass into the oocyte via the granulosa cells (Colonna and Mangia. 1983).

Alanine and tyrosine were found to be at a lower level in the IO group in Table 2, and proline, pyruvic acid and arginine may be at a lower level in the IO group by pathway analysis. Since the concentrations of amino acids in the plasma decreased in the IO cows, it may supplied a low level of amino acids to the oocyte, and thus affect follicular development.

Effect of nicotinic acid metabolism on follicular development

In Table 2, the level of nicotinoylglycine and 6-hydroxynicotinid acid was decreased in the IO group. Nicotinic acid is an important factor in energy metabolism and regulates the survival time and apoptosis of cells (Sinthupoom et al. 2015). In vivo, nicotinic acid can acids (NEFA) concentration (Pires and Grummer, 2007). In coinhibit the activity of adenylate cyclase (cAMPase) to reduce lipolysis and non-esterified fatty ntrast, a low level of nicotinic acid may increase the plasma NEFA concentration, causing fatty liver

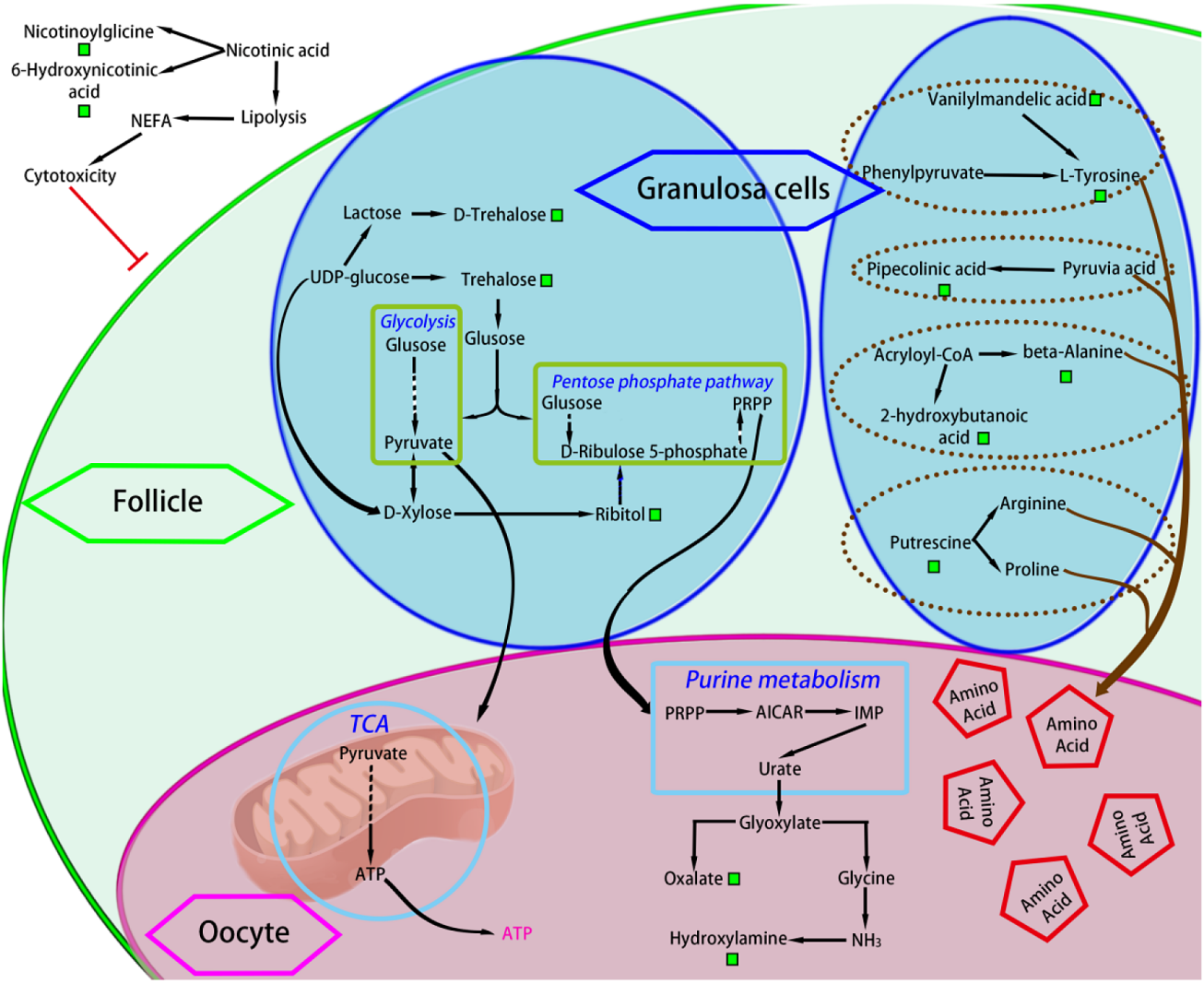

Fig. 4. Pathways involved in inactive ovaries according to pathway analysis.
Differential metabolites are marked (following or under compounds) by a green square; "→" means molecular interaction or relationship, as well as "─... →"; "─┤" means inhibition.

and increasing cytotoxicity (Cusi 2016), which inhibits follicular growth. So, it suggested that the nicotinate may play a certain role in development of follicular or inactive ovaries by nicotinamide metabolism pathway.

Conclusions

Plasma metabolic profiling obtained by GC/MS characterized the metabolites differences of dairy cows with inactive ovaries or normal function postpartum. The differential metabolites in plasma of dairy cows with inactive ovaries include ribitol, oxalate, trehalose, D-tag atose, hydroxylamine, 5-aminoimidazole-4-carboxamide ribotide, vanillylmandelic acid, phenylpyruvate, pipecolinic acid, 2-hydroxybutanoic acid, beta-alanine, putrescine, 6-hydroxynicotinic acid, nicotinoylglycine. The levels of all metabolites were lower in the inactive ovaries group than in the natural estrus group. Through pathway analysis, the plasma differential metabolites from dairy cows with inactive ovaries, which were mainly associated with carbohydrate metabolism and amino acid metabolism, may affect follicular growth. It showed that follicular growth and development in dairy cows is related to carbohydrate and amino acid metabolism by a single or multiple pathway(s).

Acknowledgements

This work was supported by the National Science Foundation Committee of China (Grant No: 31772804), the National Key R&D Program of China (2017YFD0502200), Heilongjiang Provincial Key Laboratory Prevention and Control of Bovine Diseases (PCBD201703).

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