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Proteomic analysis of the effects of lutein on mammary gland metabolism in dairy cows

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The aim of the research reported in this Research Communication was to identify differentially expressed proteins in dairy cows with normal and lutein diet and to elucidate the mechanisms of lutein-induced effects on bovine mammary gland metabolism using a comparative proteomic approach. Thirty-three differentially expressed proteins were identified from mammary gland of control diet-fed and lutein diet-fed dairy cows. Among these proteins, 15 were upregulated and 18 were downregulated in the lutein group. Functional analysis of the differentially expressed proteins showed that increased blood flow, depressed glycolysis, enhanced lactose anabolism, decreased fatty acid oxidation and up-regulated beta lactoglobulin expression were connected with lutein addition. These results suggested that the increased blood flow, reduced glucose catabolism, enhanced capacity for milk lactose synthesis, depressed fatty acid catabolism and increased expression of antioxidantion related protein may be the prime factors contributing to the increased milk production and enhanced immune status in lutein-fed dairy cows. This study provides molecular mechanism of dietary lutein in regulating lactation of dairy cows.

Keywords: Lutein, proteomics, mammary gland, dairy cows.

Lutein, one species of the carotenoid, is abundant in fruits and vegetables, such as bananas, potatoes and chrysanthemum (Mangels et al. 1993). A great number of biological functions have been claimed for lutein, including preventing oxidant-induced lipid peroxidation (Wang et al. 2013), enhancing immunity (Moraes et al. 2016) and protecting the eye against light-induced oxidative damage (Bernstein et al. 2001). Lutein has been widely used as a feed additive in livestock production. In chicken, lutein has been fed as an additive to improve laying performance and deepen the yolk color (Levy, 2001). In dairy cows, our research demonstrated that dietary lutein could enhance milk production and improve health status (Xu et al. 2014). However, the effects of dietary lutein on the mammary gland metabolism in dairy cows remain unknown.

Proteomics approach is an effective tool to profile various tissue proteins. In this study, we utilized a 2-DE/MS proteomics approach to reveal the impacts of lutein on protein profiles of the mammary gland in dairy cows and then try to understand the possible mechanisms of dietary lutein in regulating lactation of dairy cows.

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Materials & methods

Experimental animals and protein sample preparation

Husbandry details of healthy lactating Holstein cows have been described previously (Xu et al. 2014). Six dairy cows were chosen from groups given 0 or 200 g lutein preparation/d per head, according to our previous results that milk yield of dairy cows with 200 g lutein addition was the highest (Xu et al. 2014). AbsoLUTEIN, was provided by Kemin Industries (Zhuhai, China). A portion of mammary gland was collected after the cows were killed, washed with ice-cold PBS and then snap-frozen in liquid nitrogen. The mammary gland samples were prepared as described before (Wu et al. 2009). Protein concentrations were measured with Bradford protein assay kit (Sangon Biotech) and bovine serum albumin as standard according to the manufacturer's protocol. The supernatant was stored at −80 °C for later process.

Two-dimensional gel electrophoresis and image analysis

Two-dimensional gel electrophoresis was operated as formerly described (Wu et al. 2009). Mammary gland protein samples (100 μg) were mixed with rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 50 mM

DTT, 0·2% pharmalyte 3/10, and 0·005% bromophenol blue and then loaded to 24 cm, pH 3–10 nonlinear immobilized pH gradient strips in the PROTEAN IEF cell apparatus (Bio-Rad). After rehydration at 30 V for 12 h, the isoelectric focusing program was carried out with the following parameters: 500 v 1 h; 1000 v, 1 h; 8000 v, 8 h; 500 v, 4 h. After equilibration, the strips were placed on 12·5% SDS-polyacrylamide gel for electrophoresis. The gels were stained with silver stained method and were visualized using scanner (UMax Powerlook 2110 XL, GE Amersham). Three cows were performed for biological replications and three gels were run in each group for technical replication. Spot detection, spot matching and quantitative intensity were assessed automatically with Imagemaster 2D software supplied by the manufacture. The quantity of each spot was normalized by total protein quantity and data were analyzed by Student's *t*-test. Differentially protein spots ($P < 0{\cdot}05$) with a greater than 2·0-fold change were subjected to identification by MS.

In-gel digestion and protein identification

The steps were performed as previously described (Wu et al. 2009). The interesting protein spots were excised and washed, then dried in a SpeedVac (Eppendorf, Hamburg, Germany) under vacuum. The gel was digested by trypsin (Promega, Madison, America) and the digestion was terminated by 1% v/v trifluoroacetic acid. The protein spots were lyophilized. Subsequently, the peptide mixtures were mixed with matrix solution CHCA (Sigma) 60% CAN and 0·1% TFA. The samples were analyzed using a 4800 Plus MALDI TOF/TOF™ analyzer. Parent mass peaks with 800–4000 Da of mass range and 50 of minimum S/N were selected for tandem TOF/TOF analysis. A protein match with a score greater than 70 were considered statistically significant and confidence interval ⩾95% were accepted.

Bioinformatic analysis

The pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps.

Statistical analysis

All experiments were performed with three replicates. Data were statistically analyzed by the SAS software (SAS Institute, Car, NC, USA) using ANOVA with Turkey's multiple range tests. $P < 0{\cdot}05$ was considered as a significant difference.

Results and discussion

Our previous results found that dietary lutein increased milk yield, lactose content, and fat content of milk and enhanced the antioxidant capacity of dairy cows (Xu et al. 2014). We hypothesized that lutein may increase milk production and improve the health status through regulating mammary gland metabolism. To screen proteins related to the effect of lutein on mammary gland metabolism, we revealed that 33 proteins were changed in lutein group (Table 1). Among these proteins, 15 proteins were upregulated and 18 proteins were downregulated (Table 1). The upregulated proteins include UDP-galactose-4-epimerase (GALE), propionyl-CoA carboxylase (PCC), beta lactoglobulin (β-LG), serum albumin, hemoglobin and delta-pyrroline-5-carboxylate dehydrogenase (P5CDH). The downregulated proteins include aldolase A (ALDOA), enolase (ENO1), acyl-coenzyme dehydrogenase (ACADM) and acyl-coenzyme acyltransferase 1 (ACAA1). The altered proteins were related to glucose metabolism, fatty acid metabolism and immune function of dairy cows (Fig. 1).

Bovine serum albumin is the most abundant protein in the bovine blood plasma, and maintains the stability of osmotic pressure and pH of blood (Carter & Ho, 1994). Moreover, it can transport many compounds in blood including fatty acids, metal, amino acids, steroids and drugs (Carter & Ho, 1994). Bovine hemoglobin is the iron-containing oxygen-transport protein in the red blood cells (RBC) and it makes up about 35% of RBC content (Weed et al. 1963). It plays a major role in transporting oxygen to tissue and provides energy in cell metabolism (Weed et al. 1963). Our previous study found that milk yield of dairy cows was increased in lutein group, which may due to the increased expression of serum albumin and hemoglobin, indicating that lutein could increase blood flow rate to increase milk output.

ENO1 and ALDOA are two key enzymes involved in glycolytic pathway (Zommara et al. 1998) ENO1 contributes to catalyzing the formation of phosphoenolpyruvate from 2-phosphoglycerate (Zommara et al. 1998). ALDOA converts fructose-1, 6-biophosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Zommara et al. 1998). In the present study, the downregulation of ENO1 and ALDOA is likely to inhibit the glycolysis process resulting in more glucose in blood that is the major precursors for milk lactose synthesis. Besides, the increased GALE in lutein group could catalyze the interconversion of UDP-galactose and UDP-glucose in the process of lactose synthesis (Chen et al. 1999). Therefore, we speculate that lutein in diet may generate more glucose and then transfer it from blood to mammary gland, leading to the increase of milk lactose synthesis in dairy cow.

Milk fat content and composition, important indicators measuring the quality of milk, are significantly influenced by diet (Bauman & Griinari, 2003). Triglyceride is the dominating component of milk fat and is synthesized from free fatty acid in mammary gland. In our proteomic analysis, proteins associated with fatty acid metabolism including PCC were upregulated, whereas ACAA1 and ACADM were downregulated. PCC is a multimeric protein localized to the mitochondrial matrix which can function in the catabolism of isoleucine, threonine, methionine, valine, and odd-chain fatty acids (Browner et al. 1989). ACAA1,

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Table 1. Identification of differentially expressed mammary gland proteins

No.†	Spot number‡	Protein name	Accession no.	Matched peptides§	Score	Mw (Da)	pI	Change
Milk lactose metabolism								
1	1538	UDP-galactose-4-epimerase	gi|296490070	16	403	38628·4	6·75	↑
2	1435	Aldolase A	gi|296473262	12	609	39924·5	8·45	↓
3	1186	Enolase;	gi|109940077	17	561	47638·5	6·37	↓
Milk fat metabolism								
4	977	Propionyl-CoA carboxylase	gi|122137078	10	397	58 901	7·14	↑
5	1353	Acyl-CoA dehydrogenase,	gi|122144044	21	973	46 942·9	8·31	↓
6	1472	acyl-CoA acyltransferase 1	gi|296478490	13	643	44 842·9	8·72	↓
Milk protein metabolism								
7	988	Tryptophanyl-tRNA synthetase	gi|194686589	6	186	52 668·4	6·44	↓
8	987	Tryptophanyl-tRNA synthetase	gi|194686589	6	305	52 668·4	6·44	↓
9	1736	Pyrroline-5-carboxylate reductase	gil 75060505	6	82	33 717·6	7·72	↓
10	1316	Ornithine aminotransferase	gi|122140925	20	831	48 444·1	6·1	↓
11	1934	kappa casein	gi|296486476	4	135	18 246·4	9·23	↓
12	895	Delta-1-pyrroline-5-carboxylate dehydrogenase	gi|193806687	19	956	62 083·6	8·37	↑
13	882	Delta-1-pyrroline-5-carboxylate dehydrogenase,	gi|193806687	15	801	62 083·6	8·37	↑
14	2068	Beta casein	gi|83406093	4	160	25 257·3	5·53	↓
Immune status								
15	2162	Beta Lactoglobulin	gi|347447467	7	269	18 441·4	4·83	↑
Milk output								
16	810	Serum Albumin	gi|367460260	34	1080	68 415·6	5·6	↑
17	2212	Hemoglobin	gi|197724897	5	497	15 043·9	8·19	↑
Others								
18	1385	Thiolase	gi|440901593	21	1230	44 788·1	8·33	↑
19	1867	Carbonic anhydrase II	gi|296480399	11	381	27 674·9	5·85	↑
20	1986	Methionine sulfoxide reductase	gi|109914952	13	544	26 029·8	7·15	↑
21	1018	Gremlin	gi|62910905	3	26	11 495·7	9·71	↑
22	1520	Phosphotriesterase	gi|263505134	17	737	39 472·2	6·06	↑
23	2146	Isoaspartyl peptidase	gi|122138764	7	265	32 372·4	7	↑
24	1613	Crystallin	gi|143811445	9	357	35 531·7	8·29	↑
25	1614	Crystallin	gi|143811445	12	612	35 531·7	8·29	↑
26	1629	Serine dehydratase	gi|296478507	8	170	34 918·2	6·1	↑
27	1502	Sorbitol dehydrogenase	gi|75069845	22	675	38 644·9	7·14	↑
28	1989	Thioesterase	gi|296489583	18	690	26 733·7	8·7	↑
29	1894	Electron transfer flavoprotein subunit	gi|91206619	6	288	27 910·2	8·24	↓
30	1242	ErbB3-binding protein 1	gi|296487441	19	379	44 174·3	6·13	↓
31	1327	Isocitrate dehydrogenase	gi|296490355	25	937	47 071·6	6·13	↓
32	1209	Keratin	gi|157427776	24	506	48 997·4	5·08	↓
33	1131	Nucleobindin 2	gi|296480105	11	248	49 328·8	5·12	↓
34	913	Phospholipase C	gi|303524	13	321	57 293	6·23	↓
35	801	Stress-induced-phosphoprotein 1	gi|122144074	32	574	63 069·5	6·08	↓
36	1519	Tropomyosin	gi|61888866	9	199	32 731·7	4·69	↓

†Represents the total proteins identified.
‡The number corresponds to the 2-DE gel in Fig. 1.
§The number of peptide identified.
↑ Represents proteins were upregulated; ↓ represents proteins were downregulated.

an acyltransferase located in peroxisome, could catalyze the first step of fatty acid β-oxidation. ACADM, one of four acyl-CoA dehydrogenases, catalyzes the initial dehydrogenation step in the mitochondrial β-oxidation of fatty acids. The downregulated proteins involved in fatty acid oxidation may result in more fatty acid by decreasing fatty acid catabolism after feeding lutein to dairy cows. Therefore, the increased milk fat in lutin group (Xu et al. 2014) may be due to the adequate substrate for milk fat synthesis.

Our previous data have revealed that lutein diet can increase the milk quality. The proteomic results have

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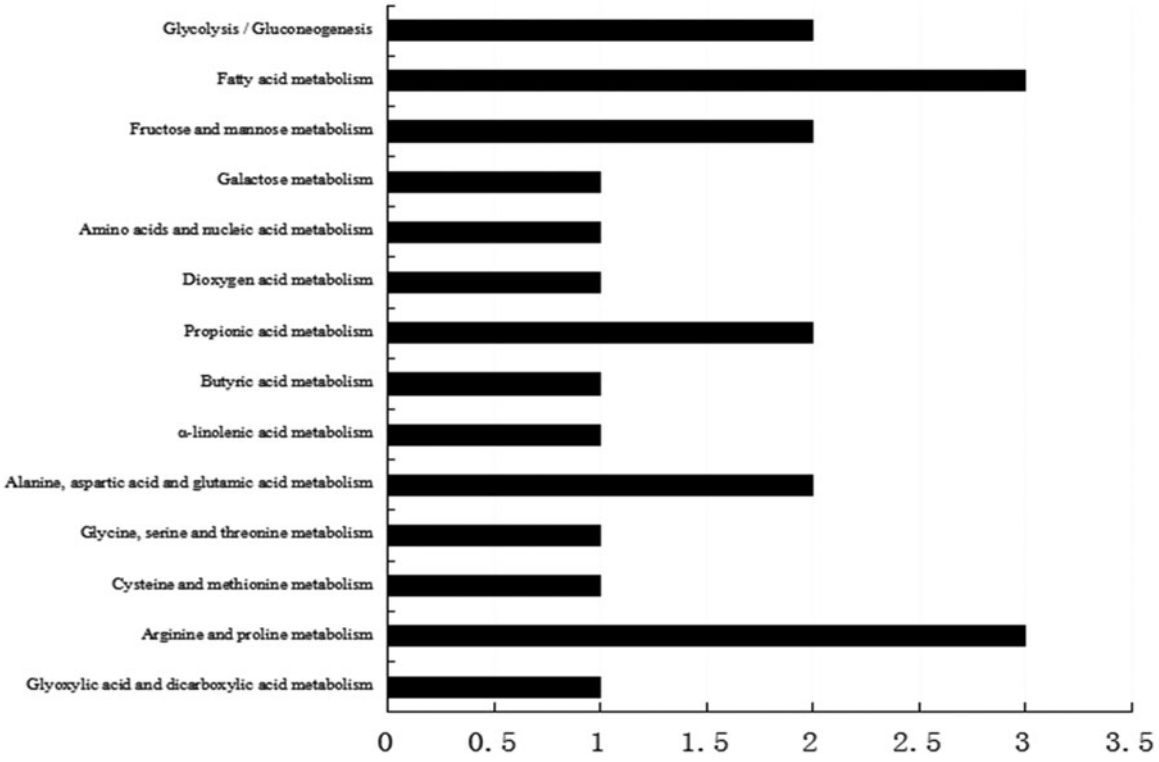

Fig. 1. KEGG pathway analysis of differentially expressed proteins.

shown that β-LG in the lutein group was increased. β-LG, the main component of bovine milk whey protein, accounts for 10–15% of the total milk and is the globular protein containing 162 amino acids (Liu et al. 2007). Beyond the nutritional contribution of β-LG, it has many biological functions including preventing lipid oxidation and increasing liver glutathione in rats (Zommara et al. 1998). The upregulation of β-LG indicated that lutein might increase the antioxidant capacity of milk and increase the milk quality.

Conclusion

A gel-based proteomics approach was used to monitor the changed protein profile of mammary gland in lutein-fed dairy cows. This study suggested four biological process contributing to the enhanced milk production and improved health status in lutein-diet fed dairy cows: enhanced blood flow, decreased glycolysis and increased lactose anabolism, depressed fatty acid oxidation, increased expression of antioxidant-related proteins. It provides a new clue to understanding the possible mechanisms of dietary lutein in regulating dairy cow lactation.

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