Metabolomics research on the effect of dexamethasone-induced chronic stress in serum of rabbits

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ABSTRACT
Stress is a common factor that reduces production performance. However, the physiology of stress is not fully understood. In this study, 40-day-old rabbits were injected 1.0 mg/kg BW Dexamethasone to induce stress. A metabolomic study on serum of stress-induced rabbits was performed to research the metabolism mechanism of stress by using ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS). Clear separations between stress rabbits and control rabbits were observed by principal component analysis (PCA) based on the data obtained using both analytical techniques and 90 significantly changed metabolites were identified as potential biomarkers of stress. Stress rabbits were characterised by altered levels of serum lysophosphatidylcholines, amino acids, phosphatidyl choline, and nucleosides, which were related to multiple perturbed metabolic pathways and contributed to the elucidation of the complex mechanism of stress. These were main linked to three different pathways: pyrimidine metabolism, aminoacyl-tRNA biosynthesis, and biosynthesis of amino acid. In summary, we provided a comprehensive understanding of metabolic alterations in the main stress-targeted tissues, helping to understand the potential mechanisms underlying stress.

HIGHLIGHTS
- Stress alters the metabolic composition of serum in rabbits.
- In rabbits, dexamethasone-induced chronic stress alters 90 metabolites in serum.

Introduction
In modern culture, vulnerability to uncontrollable and unpredictable stress is considered to be a normal occurrence in daily life. Animal stress refers to a set of abnormal reactions induced by stimuli in the organism, such as ambient temperature, noise, transport, feeding density, dietary nutrient concentration, and immunity during development. Numerous studies have shown that stress has a great impact on the health status, production performance, immune performance, blood biochemical indicators, meat quality, and body organs of animals (Franci et al. 1996; Marai et al. 2002; Li et al. 2009; Chiericato et al. 1995). Stress can induce the peroxidation of phospholipids as well as protein and DNA damage (Raschke et al. 2006). It also influences the metabolic levels of animals when animals are stressed, causing metabolic disorders and other metabolic diseases (Belhadj Slimen et al. 2016; Incalza et al. 2018). It has a major function, including hypertension, adiposity, and insulin resistance, in metabolic syndrome (Ceriello and Motz 2004). Furthermore, in modern rabbit farming systems, the excessive pursuit of growth rate, fur quality, and feed remuneration has made modern rabbit breeds increasingly vulnerable to different stress factors. While certain stress-related physiological and biochemical processes have been established, there are still many unanswered questions, such as how the entire biological system reacts to stress, which mostly includes small molecules, how the self-recovery phase of the body progresses after stress, and how stress-related illnesses arise.
The stimulation of the stress response relies mainly on the excitation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis and high circulating levels of glucocorticoid hormones (de Quervain et al. 1998; Atsak et al. 2016), thereby adjusting the body’s metabolism and producing the corresponding stress response. Dexamethasone (DEX), a type of glucocorticoid (GORT), can induce stress by the HPA and change metabolism in animals (Humbert et al. 2001; Gounarides et al. 2008). In rats, DEX treatment could significantly change several pathways, such as amino acid metabolism, pyrimidine metabolism, et al. (Malkawi et al. 2018). In broilers, DEX-induced stress decreased the absorption of glycylsarcosine (Chang et al. 2015).

Serum metabolites can reflect the metabolic changes in the body and are useful for monitoring metabolic processes under stress (Zhang et al. 2010). The serum is readily collectible and can directly reflect pathophysiological responses to disease. Metabolomics, based on the quantitative analysis of metabolites in biofluids and tissues of living systems, provides a powerful approach to study the multivariate metabolic responses to physiological stressors (Nicholson et al. 1999). Therefore, the purpose of this study was to investigate the characteristics of DEX-induced stress and metabolic especially for the metabolism of nutrients changes in the serum of rabbits, and to provide a scientific reference for the studies with the DEX-treated animal models.

Methods and materials

Animals

Rabbits were housed in homemade plastic cages (60 x 40 x 40 cm). Temperature and lighting were maintained according to commercial conditions (20–25 °C, 12 light/12 dark). The diets were formulated according to the recommendation of de Blas and Mateos (De.Blas et al. 1998). All rabbits had free access to feed and water during the rearing period. Agricultural University Animal Care and Use Committee (SDAUA-2017-029) and were in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

At 40 days of age, 40 rabbits (Hyla, male-female ratio of 1:1) with similar body weight (1388 ± 8.5 g) were divided into two groups: one received a subcutaneous injection of dexamethasone (DEX, 1.0 mg/kg body weight, 8:00 am per day) (Huang et al. 1997; Kil et al. 1997) for 7 d and the other received sham-treatment (Control, saline, 1.0 mg/kg body weight). After the last injection, six rabbits with similar weights from each group were fasted for 3 h and then electrically stunned and slaughtered. Then the blood (10 ml) was collected from a carotid vein and centrifuged at 3000 rpm for 10 min at 4 °C to obtain serum that was kept at −80 °C.

Metabolites extraction

Serum samples were thawed on ice. 100 µL of serum was taken and placed in an EP tube, then extracted with 400 µL of prechilled methanol, followed by vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15,000 rpm, 4 °C for 5 min.

UHPLC-MS/MS analysis

LC-MS/MS testing was conducted by Novogene Co., Ltd. (Beijing, China). LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). Samples were injected onto an Hyperil Gold column (100 x 2.1 mm, 1.9 µm) using a 16-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 12.0 min; 100% B, 14.0 min; 100–2% B, 14.1 min; 2% B, 17 min. Q Exactive series mass spectrometer was operated in positive/negative polarity mode with a spray voltage of 3.2 kV, capillary temperature of 320 °C, sheath gas flow rate of 35 arb, and aux gas flow rate of 10 arb.

Database search

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and the minimum intensity, 100,000. After that, peak intensities were normalised to the total spectral intensity. The normalised data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. And then peaks were matched
with the mzCloud (https://www.mzcloud.org/), mzVault, and MassList database to obtain the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using area normalisation method.

**Data analysis**

These metabolites were annotated using the KEGG database (http://www.genome.jp/kegg/), HMDB database (http://www.hmdb.ca/), and Lipidmaps database (http://www.lipidmaps.org/). Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed at metaX (a flexible and comprehensive software for processing metabolomics data). We applied univariate analysis (t-test) to calculate the statistical significance (p-value). The metabolites with VIP > 1 and p-value < .05 and fold change (FC) ≥ 1.2 or FC ≤ 0.833 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest which were based on Log2(FC) and – log10(p-value) of metabolites. For clustering heat maps, the data were normalised using z-scores of the intensity areas of differential metabolites and were plotted by the Pheatmap package in R language. The functions of these metabolites and metabolic pathways were studied using the KEGG database. When the p-value of the metabolic pathway <.05, metabolic pathways were considered as statistically significant enrichment.

**Results**

**Metabolite**

The aim of this research was to define the metabolic alterations induced by stress exposure (dexamethasone simulated) with regard to the control samples (DEX vs. control). A total of 18,532 ion features were detected from UHPLC-MS/MS analysis. The tight grouping of the QC samples in the PCA score plots further confirmed the high reproducibility and instrumental stability throughout the run (Figures 1(A,B)). The 622 identified metabolites were annotated by functional and classified.

**Multivariate data analysis**

The principal component analysis (PCA) model was constructed from aligned peak data from the control group and the dexamethasone-injected group. The first component explained 25.89%, 23.11% of the variance as shown in positive ion mode (POS) (Figure 2(A)) and negative ion mode (NEG) (Figure 2(B)), respectively. The results of partial least squares discriminant analysis (PLS-DA) were shown in Figures 2(C,D), the large degree of separation between the two groups of samples in the figure indicates that the classification effect is significant, the values of parameters were R2Y:0.97, Q2Y:0.66 and R2Y:0.99, Q2Y:0.76 in

![Figure 1](image_url). Principal component analysis (PCA) score plot of control, stress-control and quality control (QC) samples under the positive ion mode(POS) (A) and negative ion mode(NEG) (B). PCA based on the UHPLC-MS/MS spectra of QC samples and metabolites in serum samples obtained from the stress (DEX-injection) group and control group, A represented stress samples and B represented control samples.
Figure 2. PCA scores of total samples under the positive ion mode (A) and negative ion mode (B), respectively (n = 6). Partial least squares discriminant analysis (PLS-DA) with a 2D score plot of stress group and controls under the positive ion mode (C) and negative ion mode (D), respectively. Permutation analysis plotting R2 and Q2 from 200 permutation tests in the PLS-DA model, under the positive ion mode (E) and negative mode (F), respectively. A represented stress samples and B represented control samples.
POS and NEG, respectively. Permutation analysis of the PLS-DA model was also performed under positive and negative ion modes (Figures 2(E,F)). The results showed that the PLS-DA model fitting was valid and unlikely to be established by chance. The results showed that the experimental data were highly reliable.

**Significantly different metabolite**

For all identified metabolites, we identified metabolites that differed significantly between the two groups using VIP score using the PLS-DA model, \( p \)-value based on Student’s t-test were calculated, and FC (Foldchange). The criteria were \( FC > 1.2 \) or \( FC < 0.833 \), \( p \)-value < .05, and VIP > 1. A total of 90 significantly changed endogenous metabolites related to stress exposure are listed in Tables 1, 2. Results of the Volcano plot were shown in Figure 3, and each point in the figure represented a differential metabolite. For stress rabbits (DEX injected), as compared with the control group, there were 16,18 metabolites significantly up-regulated and 33,23 metabolites significantly down-regulated in POS (Figure 3(A)) and NEG (Figure 3(B)). Lysophosphatidylcholine (LPC) was significantly changed in stress-rabbits compared with the control group rabbits. Moreover, the quantities of the differential metabolites were plotted in a heatmap using hierarchical clustering analysis, as shown in Figure 4.

**KEGG functional pathway**

To explore the involved pathways of these altered plasma metabolites, KEGG pathway enrichment
analyses were performed according to the altered metabolites under both positive and negative ion modes. As shown in Figure 5, some essential pathways were detected, including pyrimidine metabolism, aminoacyl-tRNA biosynthesis, and biosynthesis of an amino acid (p-value < .05).

**Discussion**

Our previous studies have demonstrated that a chronic stress model for rabbits is established (Liu et al. 2020). Based on this model, we used a metabolic approach to reveal the mechanism underlying the change of rabbits to chronic stress and identify the biomarkers in serum monitoring stress. The serum is an optimal target as it is readily available with minimal stress for animals and is the primary carrier of various metabolites whose levels are susceptible to stress (Arimura et al. 1976; Ippolito et al. 2014). In this study, we investigated the metabolic abnormalities linked to chronic exposure to DEX in serum using the LC-MS/MS approach. Glucocorticoids, an important terminal regulator on the HPA axis, triggers a series of physiological responses that mobilise and redistribute the logical responses that had already been established...
body’s stored energy and inhibit some essential functions (e.g. growth and development) (Lv et al. 2018), thus the body develops in a direction conducive to survival and balance internal energy (Schreck 1993).

Pathway analysis was performed on the featured metabolites to identify the most relevant pathways involved in DEX administration. Pyrimidine metabolism, aminoacyl-tRNA biosynthesis, and biosynthesis of the amino acid were the most significantly enriched pathways with different impacts as shown in Figure 5. The importance of pyrimidines lies in the fact that they are structural components of a broad spectrum of key molecules that participate in diverse cellular functions, such as the synthesis of DNA, RNA, lipids, and carbohydrates. Pyrimidine metabolism is not only an important pathway to maintain appropriate pyrimidine pools in cells, but also the products of pyrimidine degradation produce diversified molecular precursors for other biological molecules, such as pantoptic acid, vitamin B5, and neurotransmitters (Tiedje et al. 2010).

In our study, we identified that deoxycytidine, cytidine, thymine, and 5-methylcytosine degraded by pyrimidine were decreased in the serum of stress rabbits. As it ranks first in KEGG enriched pathways in POS, pyrimidine metabolism may be involved in stress.

In the process of protein translation, a specific amino acid is catalysed by aminoacyl-tRNA synthetase to bind to the 3’ end of the corresponding tRNA molecule through a high-energy bond to form aminoacyl-tRNA. Therefore, the biosynthesis of aminoacyl-tRNA is important for amino acid translocation during protein translation (McClain 1993). The aminoacyl-tRNA pathway and aminoacyl tRNA synthetase enzymes are key factors required for protein biosynthesis (Ibba and Soll

| Name | Formula | Molecular weight | RT, min | FC | p-Value | VIP | Regulated |
|------|---------|------------------|---------|----|---------|-----|----------|
| LPC 18:3 | C26 H48 N O7 P | 577.34 | 14.18 | 2.19 | .0001 | 1.76 | Up |
| 1,5-Anhydro-D-glucitol | C6 H12 O5 | 224.09 | 1.31 | 0.24 | .0001 | 3.03 | Down |
| LPA 19:2 | C22 H41 O5 P | 530.33 | 14.50 | 1.84 | .0027 | 1.33 | Up |
| Pi (16:0/18:2) | C45 H81 O13 P | 860.54 | 15.37 | 0.45 | .0027 | 0.84 | Up |
| L-Histidine | C6 H9 N3 O2 | 155.07 | 1.65 | 0.65 | .0300 | 1.77 | Up |
| Pi (18:1/20:4) | C47 H81 O13 P | 884.54 | 15.33 | 0.52 | .0322 | 1.57 | Down |
| Shikimic acid | C7 H9 O5 | 155.07 | 1.65 | 0.48 | .0030 | 1.77 | Down |
| LPA 18:3 | C28 H50 N O7 P | 543.33 | 14.42 | 2.11 | .0013 | 1.74 | Up |
| DL-[β]-Leucine | C6 H13 N O2 | 131.09 | 2.14 | 0.71 | .0155 | 1.67 | Up |
| Cholesteryl sulphate | C27 H46 O4 S | 466.30 | 14.66 | 1.91 | .0409 | 1.35 | Up |
| D-(-)-Fructose | C6 H12 O6 | 180.06 | 1.29 | 1.58 | .0211 | 1.02 | Up |
| Androsterone glucuronide | C5 H9 N O4 | 101.05 | 1.20 | 0.58 | .0353 | 1.16 | Down |
| Lysophosphatidic acid | C19 H32 O8 | 388.21 | 8.69 | 0.24 | .0441 | 2.84 | Down |
| DL-Mevalonolactone | C6 H10 O3 | 130.06 | 5.40 | 1.82 | .0046 | 1.28 | Up |
| LPC 20:3 | C28 H52 N O7 P | 605.37 | 14.66 | 1.91 | .0409 | 1.35 | Up |
| Ethyl 3-[4-chlorophenethyl]amino-2-cyanoacrylate | C14 H15 Cl N O2 | 278.08 | 7.31 | 0.29 | .0552 | 2.86 | Down |
| Cystine | C6 H12 N2 O4 S2 | 240.02 | 1.20 | 1.70 | .0545 | 1.25 | Up |
| Milbemectin A3 | C31 H44 O7 | 528.30 | 12.06 | 1.62 | .0555 | 1.12 | Up |
| Lysopentadecanoic acid | C22 H46 O7 P | 467.30 | 14.42 | 0.51 | .0707 | 1.53 | Down |
| Inosine-5’-monophosphate (IMP) | C10 H13 N4 O8 P | 348.05 | 1.12 | 0.51 | .0998 | 1.58 | Down |
| LPG 18:1 | C24 H47 O9 P | 510.30 | 13.88 | 2.52 | .0109 | 1.84 | Up |
| L-Gulonic-1-Carboxylic acid | C6 H10 O5 | 164.07 | 1.30 | 0.66 | .0225 | 1.02 | Down |
| Ornithine | C5 H12 N2 O2 | 132.09 | 1.95 | 0.49 | .0227 | 1.60 | Down |
| S-Sulfo-L-Cysteine | C3 H7 N O5 S2 | 200.98 | 1.19 | 1.79 | .0245 | 1.21 | Up |
| LPI 20:3 | C29 H51 O12 P | 622.31 | 13.76 | 1.75 | .0653 | 1.14 | Up |
| Resorcinol | C6 H6 O2 | 110.04 | 6.67 | 0.59 | .0285 | 1.25 | Down |
| DL-methionine sulfoxide | C5 H11 N O3 S | 165.05 | 1.76 | 0.65 | .0321 | 1.08 | Down |
| Glucose-6-phosphate | C6 H12 O6 | 164.07 | 1.30 | 0.66 | .0225 | 1.02 | Down |
| 3,5-Dihydroxybenzoic acid | C7 H6 O4 | 154.03 | 6.68 | 0.34 | .0443 | 1.49 | Down |
| FAHFA (18:1/20:3) | C38 H66 O4 | 586.49 | 14.05 | 2.18 | .0500 | 1.67 | Up |

RT: retention time; FC: fold change; VIP: variable importance for projection, one indicator reflecting the capability of the variables to explain Y; LPC: Lysophosphatidyl choline; LPA: Lysophosphatidic acid; PI: Phosphatidylinositol; LPE: Lysophosphatidyl Ethanolamine; LPG: Lysophosphatidylglycerol; PG: Phosphatidylglycerol; LPI: Lysophosphatidylinositol; FAHFA: Fatty acid esters of hydroxy fatty acids.
Among the differential metabolites identified in this study, L-Glutamic acid and L-Histidine were significantly down-regulated in the blood of chronically stressed rabbits. The expression of several serum amino acids after DEX administration was low due to its mobilisation from protein and its subsequent breakdown as a source of carbon during gluconeogenesis. This indicates that the biosynthesis of aminoacyl-tRNA in the blood is inhibited after stress, and tRNA aminoacylation is an important marker of protein synthesis, that is, chronic stress can significantly reduce the rate of protein synthesis.

Amino acids have been proved to be involved in the changes of the physiological metabolic process (Bennett and Edelman 1969). Amino acids and their metabolites are basic substrates and regulators in many metabolic pathways and have gained increased attention from more and more researchers (Emery 2013; Dixon et al. 2014). Amino acids have been found to impact the levels of monoamine neurotransmitters (NE, 5-HT, DA) (Markus 2008), as well as regulating mood and depressive-like behavioural performance (Lee et al. 2013; Strzelecki et al., 2013 ), and are closely bound up with systemic energy levels (Kugaya and Sanacora 2005). Accordingly, in our study, the results of serum metabolomics suggest that the abnormality of biosynthesis of amino acid in stressed rabbits was significantly changed. In addition, this amino acid profile supported the phenotypes of weight loss observed in these animals.

Conclusions

In brief, we generated an untargeted metabolomic profile between stress and control groups in serum samples, we identified 49 significantly altered metabolites under positive ion mode and revealed 41 significantly altered metabolites under negative ion mode. KEGG analyses revealed that the altered metabolites mostly enriched in pyrimidine metabolism, aminoacyl-tRNA biosynthesis, and biosynthesis of amino acid. Therefore, the identified metabolites could be considered as candidate biomarkers for the diagnosis decision of stress, and the involved pathways and underlying mechanisms are worth further investigation.

Author contributions

Hongli Liu, Lei Liu, and Fuchang Li planned the experiment and finalised the draft of the manuscript with consent from all contributing authors. Hongli Liu and Bin Zhang carried out the experiment, finalised, and analysed the collected data. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors declare no conflict of interest in the submission of this manuscript.
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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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