Integration of Proteomics and Metabonomics in Exploring the Protecting Mechanism of Gushukang Capsule in Osteoporosis Rats

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Abstract

Background

Gushukang (GSK) capsule is a Chinese patent medicine for the treatment of osteoporosis (OP). It has been widely used in clinics. However, the specific mechanism and target of GSK in the treatment of osteoporosis is not clear, which needs further study.

Methods

Metabolomics (GC/MS) and proteomics (TMT-LC-MC/MC) together with bioinformatics (KEGG pathway enrichment), correlation analysis (pearson correlation matrix) and joint pathway analysis (Metabo Analyst) were employed to discover the underlying mechanisms of GSK.

Results

The regulations of differential proteins Cant1, Gstz1, Aldh3b1, Bid and Slc1a3 in the common metabolic pathway of differential proteins and metabolites between GSK/OP and OP/SHAM were corrected in GSK group. The regulations of 12 metabolites (Tyramine, Thymidine, Deoxycytidine, Cytosine, L-Aspartate and so on) were differential in the common enrichment metabolic pathway between GSK/OVX and OVX/SHAM. Differential proteins and metabolites jointly regulate 11 metabolic pathways, such as purine metabolism, pyrimidine metabolism, histidine metabolism, beta-Alanine metabolism and so on.

Conclusion

GSK may protect bone metabolism in osteoporosis rats by affecting nucleotide metabolism, amino acid metabolism and immune system.

Introduction

Osteoporosis (OP) is a systemic metabolic disease characterized by low bone mass, micro-structural damage of bone tissue, decreased bone strength and increased bone fragility[1]. Its clinical manifestations are mainly pain and spinal curvatures[2]. Traditional Chinese medicine (TCM) has been used in China and other Asian countries for thousands of years[3]. At present, under the guidance of syndrome differentiation and treatment, TCM has obvious curative effect and advantages in the treatment of osteoporosis, which can better improve the side effects and safety of Western medicine. TCM formulas not only reduce bone loss by decreasing bone resorption, but also increase bone formation in the multi-component and multitarget pattern[4]. It can also regulate the overall function of the human body and relieve back pain and lumbago[5].
GSK capsule is a Chinese patent medicine for the treatment of osteoporosis. Its main effective components include epimedium, Rehmannia glutinosa, astragalus, etc. It has the effects of dredging blood vessels, tonifying kidney and Qi, strengthening bone, and so on. GSK capsule has the characteristics of convenient administration, stable curative effect, and small side effects and has been widely used in the clinic[6]. Clinical research shows that GSK is effective in the treatment of primary osteoporosis, which is characterized by the prevention and treatment of OP by adjusting the whole body function. GSK can inhibit the formation of osteoclasts and stimulate the formation of osteoblasts in ovariectomized mice[7], and significantly increase the metabolism of vitamin D and calcium[8]. BMP-Smads signaling pathway is important in bone remodeling. Another study found that GSK significantly enhanced BMP-2/Smads signal pathway and improved bone microstructure by upregulating relevant osteogenic factors in osteoporosis rats[9]. H-type blood vessels have shown the ability to induce angiogenesis and bone formation[10]. Recent studies demonstrated that GSK can enhance hypoxia-inducible factor-1 α to induce H-type vascular formation and bone formation[11].

In recent years, combined multiomics technology has been widely used in the study of various disease states[12]. Integrating multi-omics data analysis can make up for the data problems caused by data loss, noise, and other factors in single omics data analysis, integrate various interactions isolated at the gene level or protein level, various metabolic and regulatory pathways, and jointly clarify the overall state of the biological system[13]. Among them, the combined application of proteomics and metabolomics technology undoubtedly has great advantages[14]. The data of metabolonomic analysis can provide information on the exercise of molecular function for the results of proteomic research and provide clues to explore the regulatory relationship between them. The resulting protein-metabolite interaction network intuitively shows the maladjusted related pathways in the disease state or the overall changes after drug treatment. Proteomics and metabolomics provide us with large information of differential proteins and metabolites, which provides a new research strategy for the target research of traditional Chinese medicine in the treatment of diseases. However, the specific mechanism and target of GSK in OVX rats are still unclear. Therefore, based on the integrated analysis of proteomics and metabolomics, this study used GSK capsule to treat osteoporosis rats, to further explore the mechanism of GSK in the treatment of osteoporosis.

Materials And Methods

Animal model establishment

36 3-month-old SPF Sprague Dawley female rats were obtained from Shanghai Slake Laboratory Animal Co., Ltd., and raised to 6 months old in the Experimental Center of Comparative Medicine, Fujian Academy of Chinese Medical Sciences. All rats were maintained in a 12 h light-dark cycle, with controlled temperature (22–24°C) and humidity (50–60%). After one week of adaptive feeding, 36 6-month-old rats were randomly divided into GSK group (n = 12), model group (n = 12), and sham operation group (n = 12). The osteoporosis model was established by removing the bilateral ovaries of rats; the same volume of adipose tissue beside the ovary was removed in the sham operation group[15]. Intramuscular injection of
penicillin 800 thousand units / day was used to anti-infection 3 days after operation. There are 12 weeks to develop osteopenia in OVX.

**GSK treatment**

We opened up GSK capsule (Z20060270, Liaoning Kangchen Pharmaceutical Co., Ltd.) and mixed it with normal saline by gavage once a day (4.32g / (kg * d)). Sham group and model group were given equal volume of normal saline once a day for 12 weeks. After 12 weeks of treatment, Rats in each group were anesthetized with 2% Pentobarbital Sodium. The first and second lumbar vertebrae were separated and quickly put into liquid nitrogen. The left tibia was used for micro-CT.

**Micro-CT bone analysis**

Micro-computed tomography (micro-CT) scanning and morphometric analysis were performed on a Micro-CT imaging system (ZKKS-MCT-Sharp, Zhongkekaisheng Co., China) which operated at a voltage of 70 kV and an electric current of 100 µA. The voxel size after reconstruction was 10×10×10 µm. Based on the micro-CT results, three-dimensional images were reconstructed by MicroCT Reconstruction and BMD of left tibia was determined using the analysis software. The data are displayed as means ± standard error of the mean (S.E.M.). All statistical analysis was performed on Prism 8.0 (GraphPad Prism Software, USA).

**Proteomic Sample processing**

Lumbar samples were added with appropriate SDT lysate. After boiling in the water bath, the supernatant was centrifuged to extract the protein. The lumbar vertebrate proteins of every four rats were mixed into one sample for proteomic analysis. The protein was quantified by the BCA (P0012, Biyuntian, China) method. An appropriate amount of protein from each sample was trypsinized using the Filter aided proteome preparation (FASP)[16] method and peptides were quantified (OD280). 100 UG peptide segments were taken from each sample and labeled according to the instructions of TMT labeling kit (Thermo Fisher Scientific, USA).

**Liquid chromatography tandem mass spectrometry (LC-MS/MS)**

Each fractionated sample was separated by HPLC liquid phase system Easy nLC (Thermo scientific, USA) with nanoliter flow rate. After chromatographic separation, the samples were analyzed by Q-exactive mass spectrometer (Thermo scientific, USA). Positive ion detection method, parent ion scanned range 300-1800 m / Z. Mass charge ratio collection method of polypeptide and polypeptide fragments: collect 20 fragment maps (MS2 scan, HCD) after each full scan.

**Proteomic data analysis**
The original data was analyzed by LC-MS/MS mass spectrometry software Mascot 2.2 and Proteome Discoverer 1.4. The protein database is: UniProt_ Rattus_ norvegicus_36107_ 20190524; The quantitation method was based on the median quantitative value of the unique peptide. The maximum number of missed cuts is 2; The variable modifications were oxidation (m), TMT6/10 plex (y); fixed modifications include carbamide methyl (c), TMT6/10 plex (N-term), TMT6/10 plex (k); The screening criteria of credible peptides were $\leq 0.01$.

**Metabolomic Sample processing**

Weighed about 80 mg samples, added 200ul water, homogenated in MP homogenizer, vortexed 60s, added 800ul methanol acetonitrile solution (1:1, v/v), vortexed another 60s. The suspension was ultrasonicated at low temperature for 30 min twice and placed at -20 °C for 1 h to precipitate protein. Then, the suspension was centrifuged at 4 °C, 14000 RCF for 20 min. Finally, the supernatant was freeze-dried and stored at -80 °C.

**Gas Chromatography Mass Spectrometry (GC/MS)**

The samples were separated in HILIC column by Agilent 1290 Infinity LC (Agilent, USA) ultra performance liquid chromatography (UHPLC); The column temperature was 25 °C; The flow rate was 0.3 ml / min; The mobile phase consisted of A: water + 25 mM ammonium acetate + 25 mm ammonia water, B: acetonitrile; Gradient elution. QC samples are inserted into the sample queue to monitor and evaluate the stability of the system and the reliability of the experimental data. The positive and negative ion modes of electrospray ionization (ESI) were detected respectively. The samples were separated by UHPLC and then analyzed by a triple TOF 5600 mass spectrometer (AB SCIEX). The second-order mass spectrometry was obtained by information dependent acquisition (IDA) with the high sensitivity mode. Declustering potential (DP):±60 V (positive and negative modes), Collision Energy: 35±15 eV, IDA was set as follows: Exclude isotopes within 4 Da Candidate ions to monitor per cycle: 6.

**Metabolomic data processing**

The original data was converted into. mzXML format by ProteoWizard, and then peak alignment, retention time correction, and peak area extraction are performed by XCMS program. The metabolite structure identification uses accurate mass matching (<25 ppm) and secondary spectrum matching methods and searches the laboratory's self-built database. After Pareto-scaling pretreatment, the data was analyzed by multivariate statistics, including unsupervised principal component analysis (PCA), supervised partial least squares (PLS-DA) and orthogonal partial least squares (OPLS-DA). To further screen the differential metabolites, we used PLS-DA data model to obtain the model Variable Importance for the Projection (VIP) under the condition of positive and negative ion mode to screen the differential metabolites between GSK group / model group and model group / sham operation group. The metabolites with multidimensional statistical analysis VIP > 1 and univariate statistical analysis $p$ value < 0.05 were selected to identify the potential metabolites with significant differences. Subsequently, metaboanalyst 5.0 was used to analyze the metabolic pathways involved in differential metabolites and evaluate their importance. When the
impact value of metabolic pathways is greater than 0.2, we consider these pathways as potential target metabolic pathways in this experiment.

**Results**

**Micro-CT evaluation**

The proximal end of left tibia was evaluated with micro-CT plain scan and BMD (Fig. 1) to characterize the effects of treatment on bone mineral density (BMD). Compared with the sham group, the model group had significantly lower BMD ($p<0.001$), decreased number of bone trabeculae, increased Tb.Sp ($p<0.01$), and disrupted bone microarchitecture. Compared with the OVX group, the BMD of GSK was higher ($p<0.05$), and saved bone mineral loss from OVX in trabecular bone.

**Results of proteomic analysis**

The expression of differential proteins in GSK, OVX, and SHAM group were statistically analyzed. Differentially expressed proteins in OVX rats were corrected in GSK group (Top 5)(Table 1). And there were top5 up-regulation and down-regulation differentially proteins in OVX group (Table 2).

| Uniprot ID | Protein name                           | Gene name | GSK VS OVX | P value | Difference multiple | Regulation |
|------------|---------------------------------------|-----------|------------|---------|---------------------|------------|
| A0JPP1     | Dr1-associated corepressor            | Drap1     | 0.026      | 1.749   | Up                  |            |
| G3V7U0     | Cysteine and glycine-rich protein 3   | Csrp3     | 0.048      | 1.527   | Up                  |            |
| D3ZES7     | Plexin A4                             | Plxna4    | 0.044      | 1.404   | Up                  |            |
| M0RDF2     | Ig-like domain-containing protein      |           | 0.031      | 1.392   | Up                  |            |
| D4A7U8     | Myozenin 1                            | Myoz1     | 0.041      | 1.383   | Up                  |            |
| Q5BJX0     | N-terminal Xaa-Pro-Lys N-methyltransferase 1 | Ntmt1  | 0.003      | 0.833   | Down                |            |
| P0C0R5     | Phosphoinositide 3-kinase regulatory subunit 4 | Pik3r4   | 0.026      | 0.833   | Down                |            |
| Q5U206     | Calmodulin-like protein 3             | Calml3    | 0.008      | 0.833   | Down                |            |
| P70582     | Nuclear pore complex protein Nup54    | Nup54     | 0.031      | 0.832   | Down                |            |
| A0A0G2K6H2 | Maleylacetoacetate isomerase          | Gstz1     | 0.021      | 0.832   | Down                |            |
Table 2
Statistics of up-regulation and down-regulation proteins between OVX and SHAM (top5)

| Uniprot ID | Protein name          | Gene name     | OVX VS SHAM | P value | Difference multiple | Regulation |
|------------|-----------------------|---------------|-------------|---------|---------------------|------------|
| D3ZAT0     | RCG32168              | Svs3b         | ≤0.001      | 6.329   | Up                  |            |
| O88753     | Epsilon 2 globin      | Hbe2          | ≤0.001      | 5.373   | Up                  |            |
| B2RYM6     | Zc3hc1 protein        | Zc3hc1        | 0.010       | 2.493   | Up                  |            |
| Q6AY07     | Fructose-bisphosphate aldolase | Aldoart2 | 0.039 | 2.284 | Up | |
| E9PTV9     | Glyceraldehyde-3-phosphate dehydrogenase | - | 0.002 | 2.071 | Up | |
| P62762     | Visinin-like protein 1 | Vsnl1        | 0.012       | 0.831   | Down                |            |
| P43278     | Histone H1.0          | H1f0          | 0.003       | 0.830   | Down                |            |
| F1LST1     | Fibronectin           | Fn1           | 0.043       | 0.828   | Down                |            |
| D3ZPV2     | RCG43880              | Tktl1         | 0.033       | 0.821   | Down                |            |
| P14480     | Fibrinogen beta chain | Fgb          | 0.004       | 0.819   | Down                |            |

Results of metabolomics analysis

The results of the principal component PCA analysis show that QC and other samples are well aggregated within the group, indicating that the overall quality of the experimental data meets the analysis requirements. There were differences in metabolite levels among GSK, SHAM, and OVX groups (Fig. 2). The significant difference metabolites mainly involved in these pathways (Fig. 3): Phenylalanine, Tyrosine and Tryptophan, Glycine, Serine and Threonine metabolism, Phenylalanine metabolism, Synthesis and degradation of ketone bodies, Beta alanine metabolism, Cystine and methionine metabolism, Pyruvate metabolism, etc.

The PCA method observed the aggregation degree and the overall distribution trend of samples within the group.

The number of metabolic pathways involved in differential protein and differential metabolites

The differential proteins in GSK/OVX group were involved in 70 metabolic pathways (Fig. 4a), the differential metabolites were involved in 73 metabolic pathways, and there are 45 pathways that the two
parts jointly participate in. The differential proteins in OVX/SHAM group were involved in 93 metabolic pathways (Fig. 4b), the differential metabolites were involved in 65 metabolic pathways, and the two parts were jointly involved in 26 pathways.

Analysis of enriched differential proteins and differential metabolites in common metabolic pathways

According to the statistics of the common metabolic pathways after GSK treatment and castration, differential proteins and metabolites jointly regulate 11 metabolic pathways, such as Purine metabolism, Pyrimidine metabolism, Histidine metabolism, beta-Alanine metabolism, Inflammatory mediator regulation of TRP channels, Platelet activation, Tyrosine metabolism, Pathways in cancer, Phenylalanine metabolism, Glutamatergic synapse, Gap junction and so on (Fig. 5 and Table 3)
Table 3
Differential proteins and metabolites enriched by the common metabolic pathways in GSK/OVX and OVX/SHAM. *It is a protein or metabolite with differential expression trends between GSK / OP and OP / SHAM.

| No. | Metabolic pathway | Enriched protein | Enriched metabolites |
|-----|-------------------|------------------|----------------------|
|     |                   | GSK/OP | OP/SHAM | GSK/OP | OP/SHAM |
| 1   | Purine metabolism | *Cant1 | ↓       | *Cant1 | ↑       | L-Glutamine | ↓       |
|     |                   | Prps   | ↑       | Enpp3  | ↑       | Inosine     | ↑       |
|     |                   |        |         |        |         | Adenosine   | ↓       |
| 2   | Tyrosine metabolism | *Gstz1 | ↓       | *Gstz1 | ↑       | *Tyramine   | ↓       |
|     |                   | *Aldh3b1 | ↑       | *Aldh3b1 | ↑       | Maleic acid | ↑       |
|     |                   |         |         |        |         | *Tyramine   | ↑       |
| 3   | Pyrimidine metabolism | *Cant1 | ↓       | *Cant1 | ↑       | Dihydrouracil | ↓       |
|     |                   |        |         |        |         | *Thymidine  | ↑       |
|     |                   |        |         |        |         | L-Glutamine | ↓       |
|     |                   |        |         |        |         | *Thymine    | ↑       |
|     |                   |        |         |        |         | *Deoxycytidine | ↑       |
|     |                   |        |         |        |         | *Cytosine   | ↓       |
| 4   | Histidine metabolism | *Aldh3b1 | ↓       | *Aldh3b1 | ↑       | *L-Aspartate | ↓       |
|     |                   |        |         |        |         | *Ergothioneine | ↑       |
|     |                   |        |         |        |         | *L-Histidine | ↑       |
|     |                   |        |         |        |         | *L-Anserine | ↑       |
|     |                   |        |         |        |         | 3-Methylhistidine | ↑       |
| 5   | beta-Alanine metabolism | *Aldh3b1 | ↓       | *Aldh3b1 | ↑       | Dihydrouracil | ↓       |
|     |                   |        |         |        |         | *L-Aspartate | ↑       |
|     |                   |        |         |        |         | *L-Aspartate | ↑       |
|     |                   |        |         |        |         | *L-Histidine | ↓       |
|     |                   |        |         |        |         | *L-Anserine | ↓       |
|     |                   |        |         |        |         | *Ergothioneine | ↓       |
| 6   | Pathways in cancer | Calml3  | ↓       | Fn1    | ↑       | 1-Stearoyl-2-arachidonoyl-sn-glycerol | ↑       |
|     |                   | *Bid   |         | Kng2    |         | L-Malic acid | ↑       |
|     |                   | Cdk4   |         |         |         |             | ↑       |
| No. | Metabolic pathway                        | Enriched protein | Enriched metabolites                          |
|-----|-----------------------------------------|------------------|-----------------------------------------------|
|     |                                         | GSK/OP OP/SHAM   | GSK/OP OP/SHAM                                 |
| 7   | Platelet activation                     | Src ↓            | Rlc-a ↑                                       |
|     |                                         | Fgb ↓            | *Arachidonic Acid (peroxide free) ↑           |
|     |                                         |                  |                                               |
|     |                                         |                  |                                               |
|     |                                         |                  |                                               |
|     |                                         |                  |                                               |
|     |                                         |                  |                                               |
| 8   | Inflammatory mediator regulation of TRP channels | Calml3 ↓         | Kng2 ↓                                       |
|     |                                         |                  | *Arachidonic Acid (peroxide free) ↑           |
|     |                                         |                  | 1-Stearoyl-2-arachidonoyl-sn-glycerol ↑       |
|     |                                         |                  | Prostaglandin I2                              |
|     |                                         |                  |                                               |
| 9   | Phenylalanine metabolism                | *Aldh3b1 ↑       | Hippuric acid                                 |
|     |                                         |                  | *Benzoic acid                                 |
|     |                                         |                  | *DL-Phenylalanine                             |
|     |                                         |                  |                                               |
| 10  | Glutamatergic synapse                   | *Slc1a3 ↑        | L-Glutamine                                   |
|     |                                         |                  | 1-Stearoyl-2-arachidonoyl-sn-glycerol ↑       |
|     |                                         |                  | L-Glutamate ↑                                 |
| 11  | Gap junction                            | Src ↓            | Tubb2a ↓                                      |
|     |                                         |                  | 1-Stearoyl-2-arachidonoyl-sn-glycerol ↑       |
|     |                                         |                  | L-Glutamate ↑                                 |

**Correlation analysis of significant difference proteins and significant difference metabolites**

Based on the Pearson correlation method, the correlation coefficient between significant difference proteins and significant metabolites was calculated, and the metabolites and proteins with significant differences at the key nodes in the network were screened. We constructed a correlation network of proteins and metabolites (|R| ≥ 0.5 and p < 0.05) (Table 4). We found that the metabolites in Histidine metabolism and β-Alanine metabolism were strongly related to the differential proteins. In the correlation network of GSK/OP, seven proteins and eight metabolites as nodes included two correlations and six negative correlations (Fig. 6a). In the correlation network of OP/SHAM, two proteins and two metabolites as nodes included two negative correlations (Fig. 6b).
Table 4

Correlation statistics of GSK/OVX and OVX/SHAM significant differential proteins and metabolites

| Group     | Protein  | Metabolites                                      | Coefficient | P value | Lable |
|-----------|----------|--------------------------------------------------|-------------|---------|-------|
| GSK/OP    | Mecp2    | (3-Carboxypropyl)trimethylammonium cation        | 0.995       | 0.045   | pos   |
|           | Slc1a3   | L-Histidine                                      | -0.997      | 0.045   | neg   |
|           | Svs3b    | Isobutyric acid                                  | 0.995       | 0.045   | pos   |
|           | D3ZAT0   | Riboflavin                                       | -0.995      | 0.045   | neg   |
|           | Rbm27    | Purine                                           | -0.994      | 0.045   | neg   |
|           | Jchain   | 1-Oleoyl-sn-glycero-3-phosphocholine             | -0.998      | 0.045   | neg   |
|           | Prps2    | Oleic acid                                       | -0.995      | 0.045   | neg   |
|           | Aldh3b1  | 1-Stearoyl-sn-glycerol 3-phosphocholine          | -0.995      | 0.045   | neg   |
| OP/SHAM   | Tubb2a   | L-Aspartate                                      | -0.999      | 0.009   | neg   |
|           | KnG2     | DL-Methionine sulfoxide                          | -0.999      | 0.009   | neg   |

Discussion

With the increasing incidence of osteoporosis year by year, osteoporosis has become a worldwide health problem and social public problem[17]. Exploring the mechanism of GSK and other TCM can provide greater advantages and theoretical supplement for TCM treatment and prevention of osteoporosis. Clearly, proteins and small molecule metabolites are closely associated with the occurrence of diseases, thus the combination of proteomics and metabonomics are matched our attention. It has been reported that the specific mechanism of GSK in the prevention and treatment of OP has not yet been explained, and its molecular mechanism has not been clarified.

Based on the combined analysis of proteomics and metabonomics, the results showed that the expression trend of differential proteins Cant1, Gdstz1, Aldh3b1, BID, and Slc1a3 in the common metabolic pathway of differential proteins and metabolites between GSK/OP and OP/SHAM was different, that is, they were corrected in GSK group. Moreover, Cant1 was involved in purine metabolism and pyrimidine metabolism, and Aldh3b1 was involved in tyrosine metabolism, histidine metabolism, beta-Alanine metabolism and phenylalanine metabolism. After GSK treatment, Slc1a3 was surely correlated with L-histidine (P=0.045), Aldh3b1 was significantly correlated with 1-stearyl-sn-glycerol 3-phosphocholine (P=0.045). Combined with proteomic analysis, Gdstz1 and Aldh3b1 were located in the functional nodes of protein-protein interaction network. In addition, the expression trend of 12 metabolites (Tyrmine, Thymidine, Deoxycytidine, Cytosine, L-Aspartate, Ergothioneine, L-Histidine, L-Anserine, P-antothenate, Arachidonic Acid (peroxide free), Benzoic acid, DL-Phenylalanine) was differential in the common enrichment metabolic pathway between GSK/OPX and OVX/SHAM. The above metabolic pathways are
primarily involved in nucleotide metabolism, amino acid metabolism, immune system, cell process, and other signaling pathways, indicating that GSK may treat osteoporosis in rats through these.

Uric acid is the final product of purine metabolism in the human body, which is mainly decomposed by enzymes from nucleic acids and other purine compounds metabolized by cells and purines in food. Uric acid is a reducing substance in the human body, which participates in the redox reactions and scavenging oxygen free radicals, thereby inhibiting oxidative stress\textsuperscript{18, 19}. A number of studies have reported that serum uric acid (UA) is involved in the pathogenesis of OP by affecting oxidative stress and inflammatory cascades\textsuperscript{20}. Higher serum UA levels appear to be protective for bone loss in peri- and postmenopausal women\textsuperscript{21}. Cant1, a calcium-activated nucleotide, is essential for glycosaminoglycan synthesis in cartilage\textsuperscript{22}. In this study, the key differential protein Cant1 was involved in purine metabolism and was down-regulated after GSK treatment.

Amino acid metabolism is an important part of life activities. As the basic unit of macromolecular protein, it is essential for the normal metabolism of the human body\textsuperscript{23}. Previous studies\textsuperscript{24, 25} showed that Amino acid metabolism is important to osteoporosis. In the correlation analysis, the significant differential proteins and metabolites have a strong correlation with Histidine metabolism and \(\beta\)-alanine metabolism. Histidine is an essential amino acid in the human body during childhood. In adulthood, the human body can synthesize it by itself and histidine becomes a nonessential amino acid. Histamine is formed under the action of histidine decarboxylase and is a factor that regulates inflammation and allergic reactions\textsuperscript{26}. An increase in the amount of bone cortex and bone minerals was found in mice deficient in histamine synthesis, revealing that the lack of histamine may increase the bone density and bone formation of histamine deficient mice by stimulating the synthesis of calcitriol\textsuperscript{27}.

The results showed that after treatment with GSK, Aldh3b1 in the Histidine metabolism pathway was downregulated, L-Aspartate was downregulated, and Ergothioneine, L-Histidine, and L-Anserine were upregulated. \(\beta\)-Alanine plays a key role in bone metabolism, mainly by improving the production of insulin and insulin-like growth factor-1, as well as the synthesis of collagen and muscle proteins. Studies\textsuperscript{28} have shown that \(\beta\)-Alanine may be a potential biomarker of osteoporosis, and \(\beta\)-Alanine levels increase significantly in postmenopausal osteoporosis patients with low bone density. Tyrosine is an important amino acid in the body and the main raw material for the synthesis of thyroxine\textsuperscript{29}. In previous studies, it has been found that GSK may participate in bone metabolism by regulating tyrosine metabolism and differential proteins. In addition, Aldh3b1 is also involved in the metabolism of Tyrosine metabolism, Histidine metabolism, \(\beta\)-Alanine metabolism, and Phenylalanine metabolism. We speculate that Aldh3b1 may be closely related to the metabolism of amino acids. Aldh3b1 is a member of the ALDH family. Its function is related to the scavenging of reactive oxygen species\textsuperscript{30}. In postmenopausal women, the level of estrogen and antioxidants decreased with age, and the ROS accumulated in the body could not be cleared in time, which induced the occurrence of oxidative stress and led to the damage of osteoblasts and osteocytes\textsuperscript{31, 32}. ALDH can reduce oxidative stress through a variety of aldehyde metabolism\textsuperscript{33}.

In this study, the expression trend of arachidonic acid (AA) in the platelet activation pathway and inflammatory mediator regulation of TRP channels changed after GSK treatment. AA and its products play
an important role in regulating inflammatory response[34], vascular elasticity, platelet activation, or bone remodeling. AA belongs to polyunsaturated fatty acids (PUFAs), one of the indispensable free fatty acids in the human body. According to the position of the first double bond position[35], PUFAs can be divided into n-3pufas and n-6pufas, and n-6pufas mainly include linoleic acid and AA. Recent research highlights the potential role of PUFA in the inflammatory regulation of bone remodeling via several cellular pathways[36–38]. The combined use of n-3 PUFA and E2 exerted synergistic bone-protective efficacy through upregulation of RUNX2, an essential transcription factor for bone formation, as well as the suppression of bone-resorbing cytokine IL-1β[39].

**Conclusion**

This study reports the significant changes of proteins and metabolites in osteoporosis rats treated with GSK capsule. Integrated proteomic and metabolomics analysis showed that the molecular mechanism of GSK regulation might be related to Purine metabolism, Pyrimidine metabolism, Histidine metabolism, beta-alanine metabolism, Inflammatory mediator regulation of TRP channels, platelet activation, thyrosine metabolism, pathways in cancer. These metabolic pathways are mainly involved in nucleotide metabolism, amino acid metabolism, immune system, cellular processes, and other signaling pathways. This study provides a reference for the molecular mechanism of the GSK capsule in the treatment of osteoporosis. Follow-up studies will also verify the differential proteins and metabolites in these metabolic pathways, to provide a basis for this study.

**Abbreviations**

OP: Osteoporosis; GSK: Gushukang; TCM: Traditional Chinese medicine; OVX: Ovariectomy; VIP: Variable Importance for the Projection; IDA: Information dependent acquisition; PLS-DA: Partial least squares discriminant analysis; OPLS-DA: Orthogonal partial least squares; BMD: Bone mineral density; PCA: Principal component analysis; UA: Uric acid; AA: Arachidonic acid; PUFAs: Polyunsaturated fatty acids.

**Declarations**

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**Authors’ contribution**

Ruo-hui Lin wrote the first draft of the manuscript and did the formal analysis. Bing-ying Xie, Li-hua Xie, Jing-wen Huang and Sai-nan Chen investigated experiments. Xuan Chen and Juan Chen conducted the experiments or contributed reagents and tools. Sheng-qiang Li designed the research project and was responsible for funding acquisition. Ji-rong Ge administered and supervised the project. All authors read and approved the final manuscript.
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Availability of data and materials

The data analyzed during the study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

The experimental protocol was approved by the Animal Experiment Ethics Committee of Fujian Academy of Chinese Medicine. The experimental procedure followed the International Association of Veterinary Medical Editors’ Consensus of Authors’ Guide on Animal Ethics and Welfare and local and national regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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**Figures**
Figure 1

Bone plain scan and bone mineral density (BMD) of the trabecular bone in the proximal left tibia. Representative plain scan images of the proximal tibia were shown (sagittal) in (A) SHAM, (B) OVX, (C) GSK. The BMD indexes and Tb.Sp were shown as the mean±S.E.M. (n=3 per group). ***p < 0.001 vs. the sham group; **p < 0.01 vs. the sham group; #p < 0.05 vs. the ovx group.
Figure 2

PCA map of metabolomics in positive and negative ion mode. Each point in the figure represents a sample, and different colors represent different groups; the closer the samples are, the closer the expression patterns of metabolites are, and the smaller the difference between groups is.

Figure 3

Map of significant metabolic pathways involved in differential metabolites
Figure 4

Vene diagram of differential proteins and metabolites involved in the pathway of GSK / OVX and OVX / SHAM
Figure 5

Common metabolic pathways of differential proteins and metabolites in GSK/OVX and OVX/SHAM. Red is the upregulated expression of differential proteins or metabolites, green is downregulated expression of proteins or metabolites, and yellow is a metabolic pathway involved in differential metabolites and differential proteins.
Figure 6

Correlation analysis network between GSK/OP and OP/SHAM for significantly differential proteins and metabolites