

Untargeted metabolomics reveals the effect of lovastatin on steroid-induced necrosis of the femoral head in rabbits

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Abstract

PURPOSE Lovastatin is an important medicine and it shows a significant effect against glucocorticoid-induced necrosis of the femoral head. This study aimed to investigate the effect of lovastatin on preventing necrosis of the femoral head of by serum metabolomics strategy.

METHODS Adult healthy adult Japanese white rabbits were divided into three groups: control group, model group and drug group. The pathologic changes of femoral head were assessed with magnetic resonance imaging and microscope. Metabolomics based on UHPLC-MS/MS was used to analyze the collected serum sample. Data were analyzed using principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). All potential metabolites were identified by comparing with HMDB database, metlin database, lipid maps and chemspider database.

RESULTS 11 potential biomarkers were noted and identified as potential biomarkers. The change of biomarkers suggested that lovastatin on preventing necrosis of the femoral head may affect glycerophospholipid metabolism, linoleic acid metabolism, sphingolipid metabolism, alpha-Linolenic acid metabolism, pyrimidine metabolism, arachidonic acid metabolism.

CONCLUSION The study suggested that lovastatin could prevent the glucocorticoid-induced necrosis of the femoral head of rabbits. The possible reasons were closely associated with adjusting the lipid metabolism, inhibiting adipogenesis and delaying the osteocyte apoptosis.

Article Summary

Article focus

This study aimed to investigate the effect of lovastatin on preventing necrosis of the femoral head of by serum metabolomics strategy.

Key messages

Metabolomics based on UHPLC-MS/MS was used to analyze the collected serum sample. Data were analyzed using principal component analysis (PCA), partial least squares-discriminate analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA).

Strengths and limitations

Strengths

Metabolomics study based on UHPLC-MS/MS was applied to investigate the serum metabolite profiling of rabbits of steroid-induced necrosis of the femoral head. Potential biomarkers related to lovastatin for treatment of steroid-induced necrosis of the femoral head were discovered and verified, and their

metabolic pathways were discussed. Nuclear magnetism imaging and observation on histopathology were carried out.

Limitations

Firstly, the serum samples were collected from rabbits, which could provide the information of treatment for the use of lovastatin on SANFH. The samples from clinical patients needed further study. Moreover, the kinds of statins were not considered and we focused on the effect of lovastatin on SANFH. Additionally, this study gave the possible reason why lovastatin could prevent the SANFH. But the precise mechanism still requires a further in vivo study.

1. Introduction

Glucocorticoid-induced necrosis of the femoral head is difficult miscellaneous diseases in clinical diagnose [1]. The glucocorticoid is one of the steroid and plays an important role on clinical treatment of organ grafting, systemic lupus erythematosus, rheumatic arthritis, dermatomyositis, myasthenia, but long time or excess use of the glucocorticoid could result in escalation of osteonecrosis [2–5]. Researchers have paid more attention to steroid-associated osteonecrosis in the recently years. The effect of glucocorticoid for disease treatment is remarkable. However, because of the side effect, it is restrictive for using of glucocorticoid, resulting in influencing the treatment of diseases which need chronic or excessive use glucocorticoid [6, 7]. Therefore, the effective treatment of SANFH was appealing.

The hypotheses for the pathogenesis of SANFH were complex and comprehensive, including lipid metabolism disorder, intravascular coagulation, microvascular injury, intraosseous hypertension, osteocyte apoptosis, osteoporosis, enlarged fat cells and fat embolism and so on [2–4, 8, 9]. The lipid metabolism disorder was nuclear factor. Firstly, glucocorticoids could lead to gather the blood cells within the capillaries and increase the content of blood lipid and plasma protein, resulting in tissue ischemia or hypoxia. Secondly, glucocorticoids could promote fat decomposition, inhibit fat synthesis and the use of glucose by peripheral tissue, increase cholesterol and triglyceride levels and lead to hyperlipidemia. The hyperlipidemia may lead to the formation of fat embolus in the femoral arteries and cause ischemia or necrosis. Thirdly, glucocorticoids could promote a large number of bone marrow stromal cells to transform the adipocytes, resulting in intramedullary adipose tissue accumulation, coagulation disorders and elevation of intracranial pressure. Fourthly, the accumulation of fat in liver could release fat embolism, which attached to the damaged blood vessel wall and caused microcirculation disorder [10–13]. The drug therapies for SANFH included the statins, anticoagulant and anti-osteoporotic drugs. Some anticoagulant and anti-osteoporotic drugs were reported the effect of prevention and cure for SANFH [14]. Researchers discovered that the statins could prevent the SANFH through correcting the lipid metabolism disorder [15, 16]. Yin H et al [15] believed that simvastatin could enhance the effects of multiple decompressions in preventing progression of SANFH and reducing the risk of femoral head collapse. Nozaki Y et al [16] found that pravastatin may be effective in reducing steroid-induced osteonecrosis of the femoral head. The statins were important drug for treatment of SANFH. However, the mechanism of

lovastatin on steroid-induced necrosis of the femoral head based on metabolomics has not been reported.

Metabolomics involves the researchers of entire pattern of small molecular weight compounds of endogenous or exogenous metabolites in biological samples [17]. Recent reports have suggested that metabolomics by LC-MS could research the disease mechanism and drug effect [18–25]. Especially for untargeted metabolite analysis, ultra-high performance liquid chromatography tandem mass spectrometry analysis (UHPLC-MS/MS) could obtain the comprehensive metabolic changes, which contribute to evaluate the effect of drug and explore the mechanism and [23, 26–33]. However, metabolomics study on the effect of statins for treatment of steroid-induced necrosis of the femoral head has not been reported.

In this study, metabolomics study based on UHPLC-MS/MS was applied to explore the serum metabolite changes of rabbits of SANFH administrated by lovastatin, one of the statins. Potential key biomarkers associated with lovastatin for treatment of SANFH were discovered and verified, and their metabolic pathways were discussed. Furthermore, nuclear magnetism imaging and histological examination were carried out for the phenotype proof of lovastatin effect for SANFH.

2. Materials And Methods

2.1. Chemical and reagents

Sodium pentobarbital solution, normal saline and lovastatin was obtained from the research department. Prednisolone acetate injection was purchased from the company. Ultrapure water was purified by a Milli-Q system.

2.2. Animal handling

Rabbit care and use were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of 70 healthy adult Japanese white rabbits were purchased from Animal Center of an University. After 1 week, the rabbits were gotten venous blood to analyze the biochemical index. Except the biochemical index anomaly and the accidental death, 70 healthy adult Japanese white rabbits were randomly divided into three groups, namely control group, model group and drug group. Model group and drug group were injected prednisone solution while control group was injected same volume normal saline. Drug group was administered intragastrically with lovastatin water solution every other day at a dose of 10 mg/5 mL per time throughout the whole experiment for 6 weeks. Control group and model group were administered intragastrically with the same dosages warm distilled water. Except the accidental death and the killed rabbits, the residue rabbits were gotten venous blood 1 week, 2 weeks and 3 weeks, 4 weeks, 5 weeks and 6 weeks later.

2.3. Sample collection and preparation

According to the related reference [34], the samples were taken at the specific nodes and serum samples were prepared.

2.4. Magnetic resonance imaging scan

When the rabbits were fastened and injected by 35 mg/kg 1% sodium pentobarbital solution. The coronal scan of bilateral femoral heads of all rabbits was performed with Philips Achieva 3.0T TX MRI. The indexes were as follows: 3D T₂WI VISTA (TR 2600 ms, TE 200 ms), slice thickness 1.2 mm, flip angle 90 deg and matrix (reconstruction 256 × matrix scan 256). Then the MRI results of lovastatin for treatment of SANFH were researched [34].

2.5. Histological Examination

The femoral heads of rabbits in the experimental groups were taken at 0 week and 6 weeks after experiment process. The femoral heads were prepared by washing, decalcification, paraffin embedding and HE staining [3]. The signal acquisition and analysis system from optical microscope (Olympus, Japan) was used for observation, analysis and assessment.

2.6. UHPLC-MS/MS analysis

UHPLC-MS/MS analysis was performed using Q Exactive Plus High Resolution Mass Spectrometer (THERMO, USA) equipped with Ultimate 3000 (DIONEX, THERMO, USA). Acquity™ UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters, USA) was used. The chromatographic condition in reference was used [34].

2.7. Statistical analysis and metabolite identification

The raw data were acquired using Xcalibur Qual Browser (THERMO, USA). The data were imported into the Progenesis Q1 (Waters, USA) for data analysis. Pre-treatment procedures, such as peak finding, alignment, filtering and normalization were performed to process the raw data. Principal component analysis (PCA) was used to obtain an overview of variations among the different groups. For the multivariate analysis, partial least squares-discriminate analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were conducted with EZinfo 3.0 software. For LC/MS datasets, peaks were preliminarily identified by referring to online databases (HMDB, METLIN, Basic lipid, Glycerol lipid and Lipid MAPS). The results were matched with the molecular and fragment ions of experiment MS/MS spectra as well as confirmed with that of literatures [29, 30].

The Heml 1.0.3.7 software packages were used to make a heat map to show the relative amount of differential metabolites and to give their hierarchical cluster analysis (HCA) [34, 35]. The pathway analysis of the dataset of identified key metabolites was also performed using MetaboAnalyst[34]. The identified key metabolites at different time of trial groups were shown in the histogram and were proceeded normality test and one-way ANOVA by SPSS software (Ver. 19.0) (IBM, USA). Differences were considered significant at $P < 0.05$.

2.8. Quality control

For validating the stability of the analysis process, quality control sample (QC) was first prepared by mixing serum from each sample (10 μ L)[34]. QC was injected ten times before the batch process during the analysis process, to monitor the stability of sample preparation and instrument.

3. Results

The intuitive experimental flow graph of the overall serum metabolomics approach on drug effect in this study was shown in Fig. 1.

3.1. Multivariate data analysis

A pooled quality control (QC) sample was repeatedly analyzed during sample runs to evaluate the analytical stability of LC-MS based methods for global serum metabolic profiling. The overlapped total ion current (TIC) chromatograms of the QC sample demonstrated the strong repeatability of our LC-MS system (Fig. 2A). Typical TIC chromatograms of the serum metabolic profiles of the control sample, model sample and drug sample analyzed by LC-MS were shown in Fig. 2B. Obvious separation trend between control sample and the different time of drug sample was observed in Principal component analysis (PCA) score plot (Fig. 3A). Subsequently, partial least squares-discriminate analysis (PLS-DA) was applied to the classification of control sample and the different time of drug samples (Fig. 3B). The trend of different time points after drug administration gave an intuitive result to evaluate the therapeutic effect. After 1 week, 2 weeks and 3 weeks administration of drug, the serum sample points of rabbits were close to each other, despite they were separated. However, groups of 4 weeks, 5 weeks and 6 weeks after administration of drug could be clearly separated since the endogenous metabolites changed remarkably in these three groups. Although the PCA and PLS-DA figure provided an overview of the groups, the variables responsible for differences in each cluster were still unclear. The orthogonal partial least squares-discriminant analysis (OPLS-DA) as a supervised pattern recognition approach was used to find the metabolites. Figure 3C- Fig. 3F showed the loadings of serum different phases when model group compared with different time of drug group. Supplementary Fig. 1 showed the S-plots of serum different phases and the goodness of fit. In OPLS-DA model, the values of R^2Y and Q^2 were close to 1.0 suggested that models established in this study had good fitness and prediction.

3.2. Metabolites identification

According to the tag of S-plots and $P < 0.05$, $CV \leq 0.3$, 22 representative metabolites were detected between drug group and model group. In our study, biomarkers were tentatively identified based on accurate mass measurements via UHPLC-MS/MS, comparison with theoretical to observed isotopic patterns, MS/MS fragment or comparison with HMDB database (<http://www.hmdb.ca/spectra/ms/search>), metlin database (<https://metlin.scripps.edu>), lipid maps (<http://www.lipidmaps.org/>) and chemspider database (<http://www.chemspider.com/>). To illustrate the potential biomarker identification process, one metabolite ($t_R=19.26$ min, m/z 494.3235) was detailed as an example to be described. First of all, extracted ion m/z

494.3235 in chromatogram, the retention time 19.26 was obtained and was the same as measurement (Fig. 4A). Additionally, the assistant software packed in Xcalibur was used to determine the elementary composition for the peak at m/z 494.3234. The calculated list provided several possible element compositions when considering the elements of carbon, hydrogen, nitrogen, oxygen and phosphorous (C, H, N, O and P). The lower the error value, the better the fit. After a series of analysis, only one possible element composition of $C_{24}H_{49}NO_7P$ was obtained and it was the ion of $[M + H]^+$ (Fig. 4B). Thirdly, the element composition was compared to those registered in the databases, and the metabolite was preliminarily identified as LysoPC (16:1). Lastly, mass fragmentation experiment was conducted to confirm the identification. The fragments of m/z 86, 104, 184 and 476 were observed in MS/MS spectrum (Fig. 4C), where m/z 86.0970 stands for $[CH_2CHN(CH_3)_3]^+$, m/z 104.1073 stands for $[HOCH_2CH_2N(CH_3)_3]^+$, 184.0732 stands for $[H_2O_3PO-CH_2CH_2N(CH_3)_3]^+$. Both of them are typical fragments of PC, and moreover, 476.3133 stands for $[M-H_2O + H]^+$, further supporting the suggestion that this metabolite belongs to the class of LysoPC, all these data showed very good accordance with standard spectrum in metlin databases (Fig. 4D). Based on all the inference, the biomarker was identified as LysoPC (16:1).

3.3. The changes of the metabolic profile

Based on the protocol above, 11 potential biomarkers were identified. The change trend of potential biomarkers in different groups were shown in Fig. 5. The normalized abundance of metabolites were transformed by arcsinh function because their distributions were skewed. The arcsinh was incremental function and it could describe the trend of contents of metabolites in every group. The red column was control group, green column was model group and the blue columns were drug group at different time. After injecting the prednisolone acetate solution, the contents of some metabolites (LysoPC (14:0), LysoPC (16:1), LysoPC (22:6), PC (38:7), PC (36:1), LysoPC (18:1), PC (40:8)), tetracosahexaenoic acid and valeryl carnitine) in model group were significantly lower than those in control group ($P < 0.05$). The contents of some metabolites (LysoPC (20:4)) of model group were significantly higher than those in control group ($P < 0.05$). And it found that relative intensity of most of these main metabolites in drug 5 weeks group and 6 weeks group were closer to normal control group, while drug 1 week group, 2 weeks group and 3 weeks group were closer to model group.

3.4. Heat map of the metabolites and metabolic pathway analysis

According to the peak area of each metabolite of all groups, Heml 1.0.3.7 software packages were used to make a heat map in order to analyze these differential metabolites globally. The horizontal axis of the figure showed a dendrogram of the samples. The concentrations of different metabolites varied significantly in the different group. The vertical axis of the figure showed a dendrogram of the metabolite differences. The relative amount of metabolites in each sample could be visually discovered. Significant clustering and relevance of the metabolites was shown in the heat map (Fig. 6).

To explore the possible metabolic pathways after drug administration, we performed pathway analysis by metaboanalyst 3.0. Pathway impact plots were used to visualize the impact of altered metabolic

pathways (Supplementary Fig. 2). The biological pathway analysis revealed six main metabolic pathways, including glycerophospholipid metabolism, linoleic acid metabolism, sphingolipid metabolism, alpha-Linolenic acid metabolism, pyrimidine metabolism, arachidonic acid metabolism. Among those, the P value of glycerophospholipid metabolism, linoleic acid metabolism, sphingolipid metabolism and alpha-Linolenic acid metabolism were less than 0.05, with the impact-value 0.096, 0.077, 0.05 and 0.042, respectively, were filtered out as the most important metabolic pathways. The detail of pathways were listed in the Supplementary Table 1.

3.5. Observation on histopathology and magnetic resonance imaging scan

In the control group, there were rich hematopoietic cells, relatively fewer lipocytes and eosinophils. The bone trabeculas were regularly arrayed, with complete structure, clearly visible osteocytes and a few empty lacunae (Fig. 7A). In the model group, typical osteonecrosis was presented. Bone trabeculas turned thinner, ruptured and irregularly arrayed. Many empty lacunae were observed. The lipocytes were enlarged and there were rich lipocytes and eosinophils (Fig. 7B). In the drug group, there were a few lipocytes and eosinophils. The bone trabeculas irregularly arrayed, and only a few empty lacunae were observed (Fig. 7C).

In the control group, the signal of femoral head was normal, and there was no abnormality inside and outside the joint soft tissue (Fig. 8A). In the model group, the bilateral femoral heads slightly got collapsed and flattened. The intensity of T₂WI showed heterogeneous high-signal intensity. There were increasing hydrops in right articular cavity and the gap of bilateral hip joints were uneven. The hydrops of bilateral spatium inter muscle are increased and there were rich hydrops in subcutaneous soft tissue (Fig. 8B). In the drug group, there were no apparent signals in bilateral femoral head and soft tissue muscle were noted. The gap of bilateral hip joints were aequilate. There were a little hydrops in right articular cavity (Fig. 8C).

4. Discussion

The use of statins in the treatment of steroid-induced necrosis of the femoral head has become popular in the past years [16, 36]. As the clinical medicine, the statins have been proved that were useful in preventing steroid-induced necrosis of the femoral head in clinical treatment [15]. However, the key metabolic pathways and potential biomarkers were not clear.

Metabolomics offered the opportunity to explore the effect of mechanism of lovastatin on the treatment of SANFH. PCA and PLS-DA model could provide an overview of the groups and OPLS-DA model was used to screen out the representative metabolites. PCA analysis showed an apparently cyclic trend. Good reproducibility of total ion chromatograms of the quality control group and ideal values of R²Y and Q² could make sure the reliability of analysis. The identification is one of the crucial and difficult process of current metabolomics studies. Potential metabolites were identified based on accurate mass measurements via UHPLC-MS/MS, comparison with theoretical isotopic patterns, MS/MS fragment or

comparison with HMDB database, metlin database, lipid maps and chemspider database. Through identification, 11 potential biomarkers were screened out, mainly contained phosphatidylcholine (PC), which were the closely related to the SANFH [4, 26].

Many researchers have suggested the possible pathogenesis of steroid-induced necrosis of the femoral head, such as lipid metabolism disorder, intravascular coagulation, microvascular injury, intraosseous hypertension, osteocyte apoptosis, enlarged fat cells and fat embolism and so on [2–4, 8, 9]. Glucocorticoids could not only stimulate but also regulate the process of adipogenesis occurring in the bone marrow stroma [13]. Indeed, the hypotheses had the close relationship with each other. According to the previous studies and this study, it could conclude that the glucocorticoid-induced necrosis of the femoral head were closely related to the lipid metabolism disorder, osteocyte apoptosis and the hypertrophy and hyperplasia of the fat cells in the bone marrow. Lovastatin, as one of the lipid-lowering drug, could not only adjust the lipid metabolism but also decrease the adipogenesis in the bone marrow stroma [13]. Cui Q et al showed that lovastatin inhibited steroid induced fat specific gene expression and counteracted the inhibitory effects of steroids on osteoblastic gene expression in vitro study [37]. Lovastatin may shunt uncommitted osteoprogenitor cells in marrow from the adipocytic pathway to the osteoblastic pathway by enhancing osteoblast genes (PPAR γ 2 and 422aP) expression and by inhibiting adipogenesis [38].

Our results showed that lovastatin might contribute to prevent and cure the SANFH. The cause of the steroid-induced osteonecrosis maybe multifactorial. There may be several possible mechanisms for this effect. Firstly, the pathways of glycerophospholipid metabolism, linoleic acid metabolism, sphingolipid metabolism, alpha-Linolenic acid metabolism and arachidonic acid metabolism belonged to the lipid metabolism. Researchers have reported that the statins could cure the SANFH and the possible mechanism was correcting the lipid metabolism disorder [15, 16]. Lipid metabolism was the core metabolism and was closely associated with several hypotheses [2–4, 8, 9]. Lovastatin, as one of the statins, was the lipid-lowering drug. Some researchers found that abnormal lipid metabolism is a strong risk factor, which may be the key pathomechanism of steroid-induced necrosis of femoral head [39]. Therefore, adjusting the lipid metabolism maybe was the important reason for lovastatin treatment of SANFH. Additionally, compared with control group, the contents of 7 potential biomarkers were lower in model group and they were phospholipids, namely LysoPC (14:0), LysoPC (16:1), LysoPC (22:6), PC (38:7), PC (36:1), LysoPC (18:1) and PC (40:8). For model group, the decreased phospholipid led to the permeability of cytomembrane increased, which was occurred in the process of apoptosis. The apoptosis was closely related to the femoral head necrosis [40, 41]. After lovastatin administration group, the contents of these phospholipids increased. The degree of apoptosis maybe alleviate. The lovastatin cured the SANFH maybe had a relationship with alleviating the apoptosis. Moreover, lovastatin could prevent development of steroid-induced osteonecrosis in rabbits by inhibiting adipogenesis [13]. As the results of histopathology in this study, the lipocytes were enlarged and there were rich lipocytes and eosinophils in model group. In control group, there were rich hematopoietic cells, relatively fewer lipocytes and eosinophils. In drug group, there were a few lipocytes and eosinophils. The lipocytes were representative change in the experiment process. The steroids induced a significant enlargement of the

adipocyte size and a fat accumulation in the bone marrow, which contributes to an intraosseous hypertension and a decreased blood flow [2–4]. The results of our study support the previous research findings that the use of lovastatin with SANFH could have a potential to preserve the bone mass by adjusting the lipid metabolism, inhibiting adipogenesis and delaying the osteocyte apoptosis to decrease the incidence of osteonecrosis and it is meaningful for patients need long-term or high-dose glucocorticoid therapy.

Nevertheless, our research was novel, but there were some limitations. Firstly, the serum samples were collected from rabbits, which could provide the information of treatment for the use of lovastatin on SANFH. The samples from clinical patients needed further study. Moreover, the kinds of statins were not considered and we focused on the effect of lovastatin on SANFH. Additionally, this study gave the possible reason why lovastatin could prevent the SANFH. But the precise mechanism still requires a further in vivo study.

5. Conclusion

In conclusion, untargeted metabolomics reveals that the significant changes were showed during treatment of on steroid-induced necrosis of the femoral head in rabbits after injected the lovastatin. In the process of trial, 11 potential metabolites changed and they mainly involved in six metabolic pathways. Combined the observation on histopathology and magnetic resonance imaging scan, we inferred that lovastatin could significantly decrease the incidence of osteonecrosis in the steroid induced rabbits. The possible reasons were closely associated with adjusting the lipid metabolism, inhibiting adipogenesis and delaying the osteocyte apoptosis.

English Abbreviation

Abbreviation	Full name
SANFH	steroid-induced avascular necrosis of the femoral head
UHPLC-MS/MS PCA	ultra-high performance liquid chromatography tandem mass spectrometry analysis principal component analysis
PLS-DA	partial least squares-discriminate analysis
OPLS-DA	orthogonal partial least squares-discriminant analysis
MRI	magnetic resonance imaging
QC	quality control
TIC	total ion current
HCA	hierarchical cluster analysis
HMDB	human metabolome database
TEM	transmission electron microscopy
PC	phosphatidylcholine

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Ethics approval and consent to participate

The experimental design was approved by the ethical review committee of the Fourth affiliated Hospital of Nanchang university before the initiation of the study. And all experiments were in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Conflict of interest statement

The corresponding author states that there is no conflict in interest.

Consent for publication

All involved subjects and the authors listed have approved the publication of the manuscript.

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Authors' contributions

Ren contributed to the drafting and interpretation of the manuscript. Yu and Wang were involved in analyzing the manuscript. Fan, Shao and Chen conceptualized and designed the work. All authors read and approved the final manuscript.

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None

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Figures

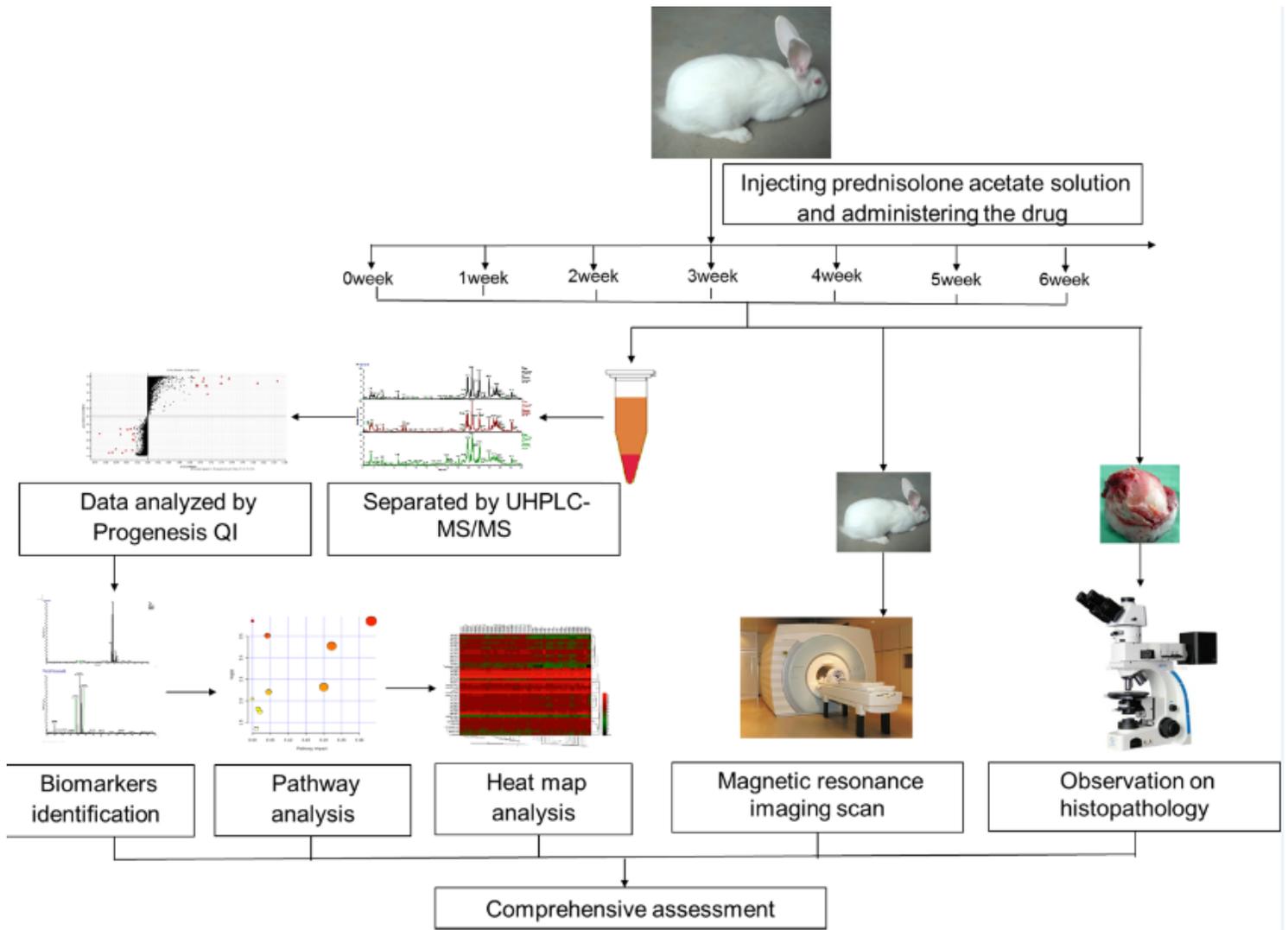


Figure 1

The intuitive experimental flow graph of the overall serum metabolomics approach on drug effect.

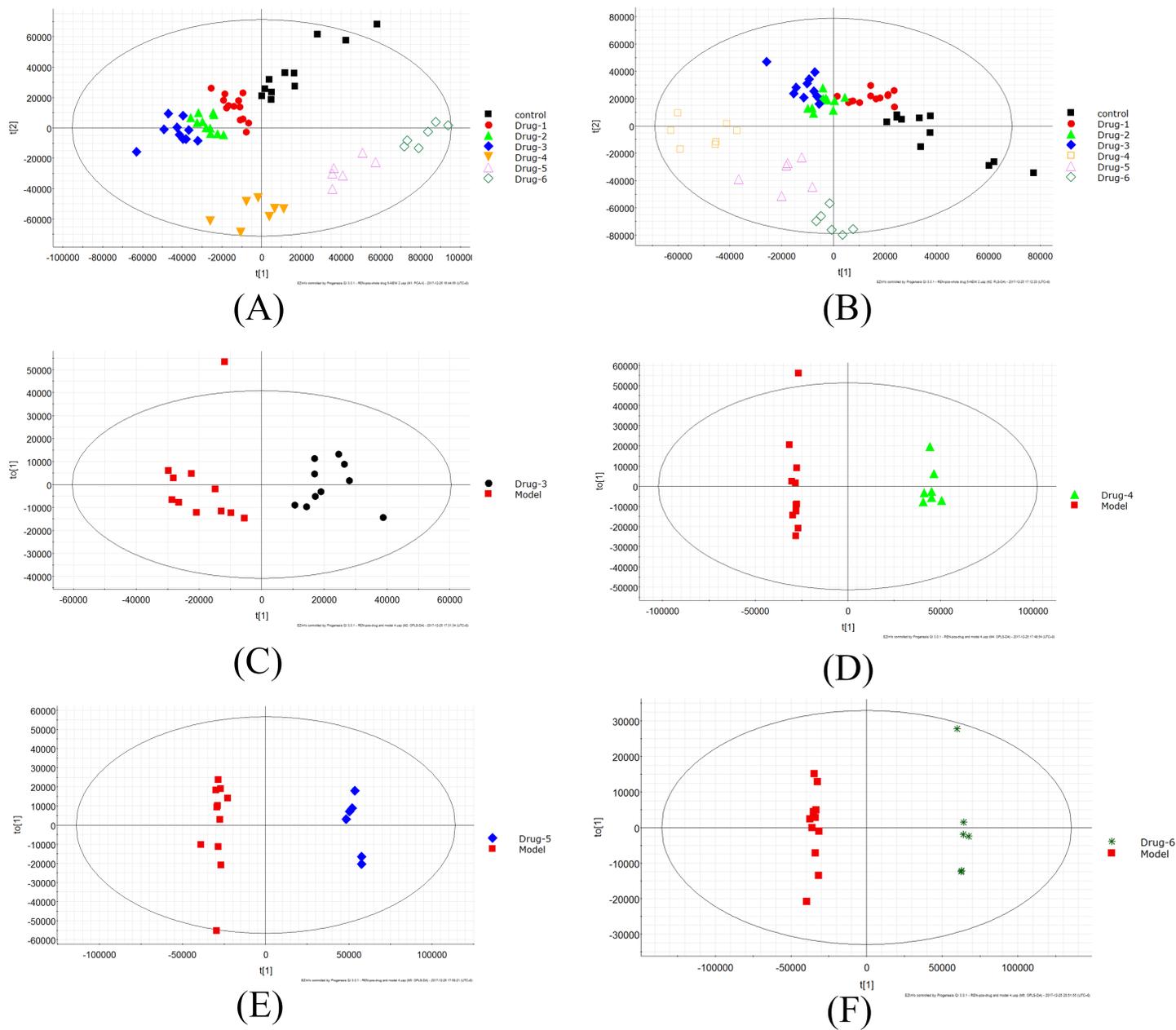


Figure 3

Obvious separation trend between control sample and the different time of drug sample was observed in Principal component analysis (PCA) score plot (Fig. 3A). Subsequently, partial least squares-discriminate analysis (PLS-DA) was applied to the classification of control sample and the different time of drug samples (Fig. 3B). Fig. 3C- Fig. 3F showed the loadings of serum different phases when model group compared with different time of drug group.

them are typical fragments of PC, and moreover, 476.3133 stands for $[M-H_2O+H]^+$, further supporting the suggestion that this metabolite belongs to the class of LysoPC, all these data showed very good accordance with standard spectrum in metlin databases (Fig. 4D).

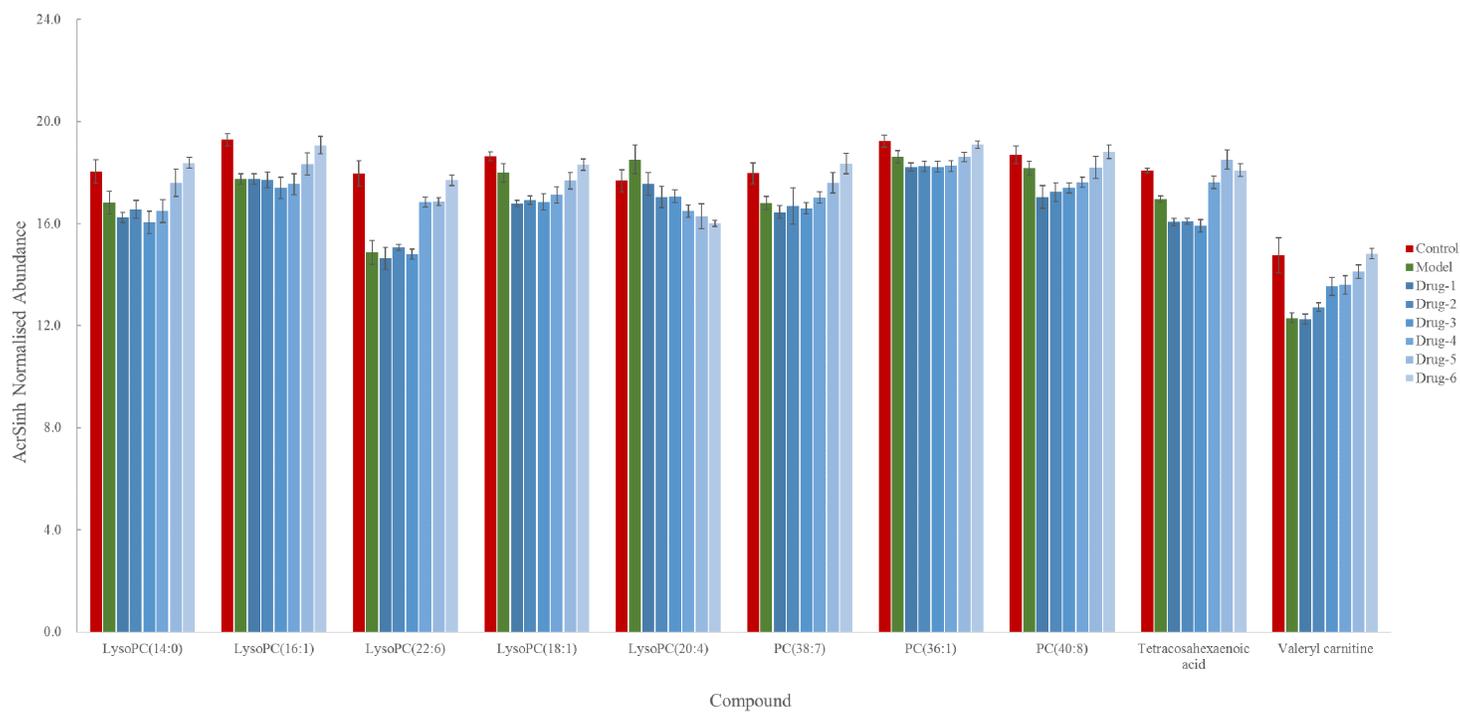


Figure 5

The change trend of potential biomarkers in different groups.

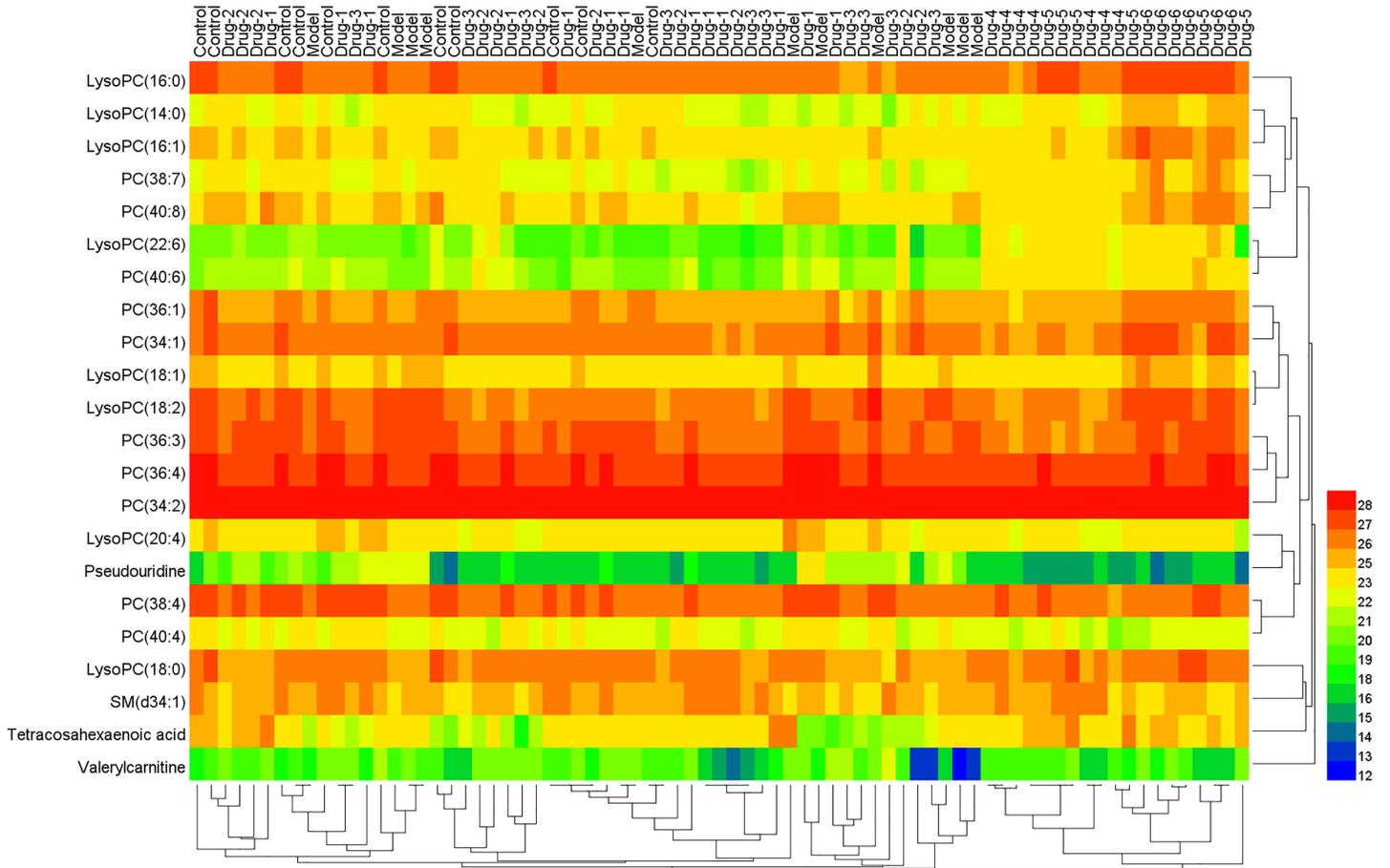


Figure 6

Significant clustering and relevance of the metabolites was shown in the heat map.

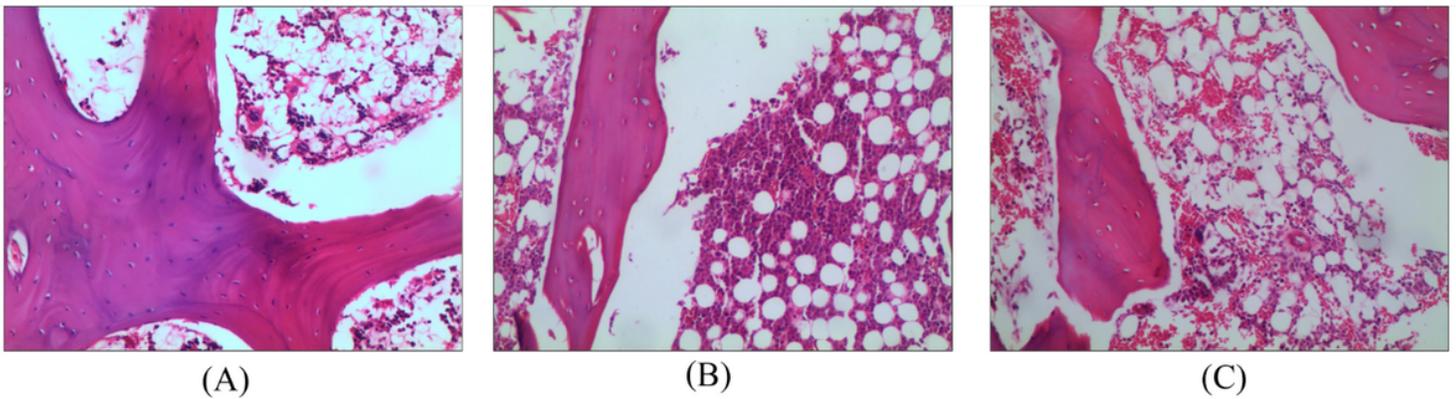
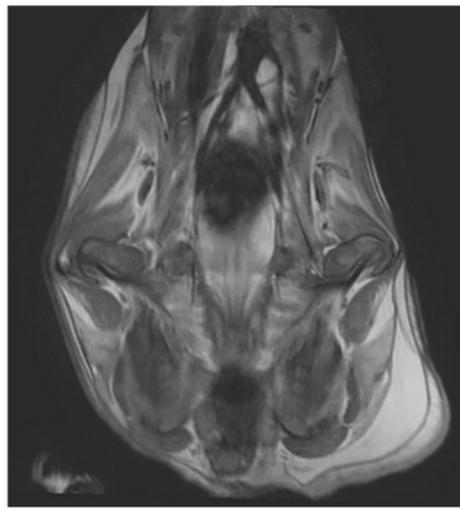


Figure 7

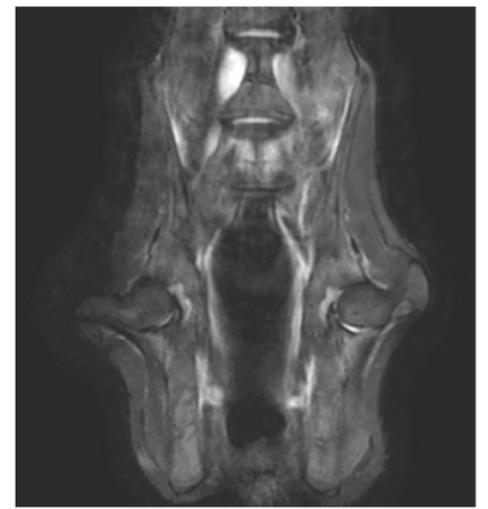
The bone trabeculae were regularly arrayed, with complete structure, clearly visible osteocytes and a few empty lacunae (Fig. 7A). The lipocytes were enlarged and there were rich lipocytes and eosinophils (Fig. 7B). The bone trabeculae irregularly arrayed, and only a few empty lacunae were observed (Fig. 7C).



(A)



(B)



(C)

Figure 8

In the control group, the signal of femoral head was normal, and there was no abnormality inside and outside the joint soft tissue (Fig. 8A). The hydrops of bilateral spatium inter muscle are increased and there were rich hydrops in subcutaneous soft tissue (Fig. 8B). There were a little hydrops in right articular cavity (Fig. 8C).

Supplementary Files

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