

LC-MS-Based Metabolomics Analysis Revealed Deleterious Effect of Infrapatellar Fat Pad on Articular Chondrocytes of Osteoarthritis

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Abstract

Objective: To investigate the interaction of infrapatellar fat pad/cartilage and related mechanisms in knee osteoarthritis (OA) using the metabolomics method.

Method: Fat-conditioned media (FCM) of the infrapatellar fat pad from patients with OA were used to treat human OA chondrocytes. The extracellular metabolites of human OA chondrocytes were detected by nontargeted metabolic footprint analysis based on liquid chromatography and mass spectrometry (LC-MS). Then, the different metabolites were found, and the main metabolic pathways were explored, combined with bioinformatics methods.

Results: After treatment with FCM for 48 h, the proliferation of human OA chondrocytes was slowed down, indicating that FCM had a certain inhibitory effect on the proliferation of human OA chondrocytes ($P = 0.023$). On the pattern diagram of principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), after FCM treatment, the data sample areas were obviously separated, indicating that FCM can significantly affect the metabolic footprint of human OA chondrocytes. Through metabolomic identification, 131 different metabolites were screened after FCM treatment compared with before treatment. For 4 pathways in total, significantly different activity levels were discovered in pairwise comparisons: alanine, aspartate, and glutamate metabolism; citrate cycle (TCA cycle); arginine and proline metabolism; and phenylalanine metabolism.

Conclusion: The infrapatellar fat pad aggravates OA chondrocyte injury and is involved in OA by disturbing the chondrocyte TCA cycle, amino acid metabolism, and glutamine metabolism, among others.

Introduction

Osteoarthritis (OA), a chronic and progressive disorder of the joints, is the most common form of arthritis [1, 2]. Epidemiological analyses have suggested that approximately 10% of the population above 60 years of age have symptomatic OA worldwide [2]. However, its pathogenesis is not fully understood. OA is now widely viewed as a disease of the whole “joint organ,” with direct and indirect interactions among multiple joint tissues (articular cartilage, subchondral bone, synovium, and periarticular adipose tissue) involved in both the maintenance and degeneration of joints [3].

As a periarticular adipose tissue located below the patella, the infrapatellar fat pad (IFP) has been confirmed to play an important role in the development of knee OA [4, 5]. To understand how the IFP might be involved in this process, the behaviors of chondrocytes have been previously examined after being cultured with IFP-derived fat-conditioned media (FCM) from OA patients, but with inconsistent results. For example, IFP-derived FCM from OA patients induced an enhanced expression of matrix metalloproteinase (MMP)1 and MMP13, as well as an enhanced collagen release from bovine cartilage, implying a deleterious effect of the IFP on cartilage [6]. On the other hand, the opposite effect was also

suggested. FCM was reported to increase collagen type II gene expression and inhibit the cell mediator in inflammatory response production (such as NO), as well as MMP1 and MMP3 gene expression [7].

In addition, these studies investigated the interaction between IFP and cartilage from the perspective of genes and protein. However, many pathway feedback mechanisms might not be reflected by the changes in protein and gene concentration. Inflammation and metabolic disorders of chondrocytes are regarded as major pathological changes in OA [8]. Acting as a secretory organ, IFP releases inflammatory mediators that participate in the local inflammation state and metabolic state of the joint during OA development [3]. However, the fundamental mechanisms of the IFP in knee OA development are largely unknown, especially at the metabolite level.

Metabolomics is a quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. It is regarded as an efficient approach for probing significant biochemical alterations and exploring potential pathophysiological mechanisms [9, 10]. It has been applied to find biomarkers and elucidate the pathogenesis of OA, with samples mainly coming from serum and synovial fluid [11, 12]. Nevertheless, studies focusing on the cell metabolism of joint components and their interaction are lacking. Hence, to better understand the effects of IFP tissue on articular cartilage, a metabolomic analysis was applied in this study to explore the functional interaction between IFP and cartilage in a coculture model.

Methods

Preparation of FCM and chondrocytes

Thirteen IFPs obtained as anonymous waste material from human subjects with OA who underwent total knee arthroplasty were used to produce FCM. The protocol was approved by the ethical committee of First Affiliated Hospital of Fujian Medical University ([2019]254). The inner parts of the fat pads, where no synovium is present, were cut into small pieces of approximately 50 mg and cultured in suspension for 24 h in a concentration of 0.3 g tissue/ml in DMEM culture medium containing 5% fetal calf serum and antibiotics. Then, the medium was replaced with serum-free DMEM (0.3 g tissue/ml) and incubated for 72 h at 37°C. After harvesting, the media were filter sterilized and stored at -80°C before use.

Primary human articular chondrocytes were derived from articular cartilage obtained from joint replacement patients diagnosed with OA (the same as those used to produce FCM). Enzymatic digestion of tissue and maintenance and culture of cells were used as previously described [13].

Chondrocyte culture

To test multiple FCM batches in one experiment, avoiding cartilage donor differences, we mixed the FCM collected several times, removed the bacteria again, and added the culture medium to prepare the conditioned medium containing 50% FCM. A total of 12 plates of chondrocytes in good condition were

cultured in the FCM and divided into OA + FCM 0 h and OA + FCM 48 h groups. Culture media were collected at 0 and 48 h, frozen in liquid nitrogen, and stored in a refrigerator at -80°C .

Cck-8 assay for cell proliferation

Cells were kept in the incubator with 5% CO₂ at 37°C after adding 10 ul of CCK-8. The microplate reader was used for measurement of optical density (OD) value, and the proliferative detection was carried out after culturing in the FCM for 0 and 48 h.

Metabolomic analysis

Culture media samples from the OA + FCM 0 h and OA + FCM 48 h groups were evaluated via ultra-high performance liquid chromatography-quadrupole/time of flight mass spectrometry (UHPLC/TOF-MS). A 100 IL aliquot of each culture media sample was added to 400 IL of methanol (containing 5 lg/mL 2-chloro-L-phenylalanine as an internal standard) and vortex mixed for 60 s. The mixture was then centrifuged at 12,000 rpm, 4 °C for 10 min, and all the supernatant was transferred to a new 2 ml centrifuge tube, concentrated, and dried in a vacuum. Then, the methanol solution was reconstituted, and the supernatant was filtered with a 0.22 μm membrane to obtain samples for testing. From each sample, 20 μL was taken to be tested and mixed into quality control (QC) samples (used to correct the deviation of the analysis results of mixed samples and errors caused by the analysis instrument itself). The remaining samples were detected by liquid chromatography-mass spectrometry (LC-MS).

Chromatography (Thermo Vanquish) was on a ACQUITY UPLC® HSS column (2.1 × 150 mm, 1.8 μm particle size) coupled with a mass spectrometer (Thermo Q Exactive Focus). The column was applied at 40 C and a flow rate of 0.25 mL/min. The mobile phases used were positive ion 0.1% formic acid water (B2)-0.1% formic acid acetonitrile (A2), negative ion 5 mM ammonium formate water (B1)-acetonitrile (A1). The gradient elution of the samples was as follows: 0~1 min, 2% A2/ A1, 1~9 min, 2%~50% A2/ A1, 9~12 min, 50%~98% A2/ A1, 12~13.5 min, 98% A2/A1, 13.5~14 min, 98%~2% A2/A1, 14~20 min, 2% A2 (ESI+)/14~17 min, 2% A1 (ESI-). The operational parameters of the mass spectrometer were spray voltage 3.5 kV (ESI+) and 2.5 kV (ESI-), sheath and auxiliary were 30 and 10 arb, respectively, for both modes. Capillary temperature was maintained at 325°C. Data were acquired in full scan mode with a resolution of 70,000. The scanning range was 81 ~ 1000, and the unnecessary MS/MS information was removed by dynamic exclusion.

Data analysis

ProteoWizard software (v3.0.8789) was used to convert the obtained raw data into mzXML format (xcms input file format). The converted data were imported into the XCMS package of R (v3.3.2) for calculation of the normalized peak intensity, exact mass, and retention time. Subsequently, these data were employed in the final dataset for multivariate statistical and pattern recognition analysis.

Statistical data analysis was performed using R (v3.3.2), which was used for the principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal projection to latent

structures-discriminant analysis (OPLS-DA). The SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the normalized integral values to determine significant differences between metabolic changes. The variables with a variable importance in project (VIP) >1 in the OPLS-DA model and $p < 0.05$ in the t-test were considered potential biomarkers. Potential biomarkers were interpreted using the Human Metabolome Database (HMDB), Metlin (<http://metlin.scripps.edu>), massbank (<http://www.massbank.jp/>), LipidMaps (<http://www.lipidmaps.org>), and mzcloud (<https://www.mzcloud.org>). Metabolomic pathway analysis (MetPA) was performed with R (v3.3.2) to identify the affected metabolic pathways and facilitate further biological interpretation. SPSS 22.0 was used for the statistical analysis.

Results

Effects of FCM on OA chondrocyte proliferation

The effects of FCM on the proliferation of OA chondrocytes was examined by performing a CCK-8 assay, and the results suggested that FCM could decrease the viability of OA chondrocytes (Fig. 1). After coculturing with FCM, the viability of OA chondrocytes decreased significantly when compared with the control ($P = 0.023$). It appears that FCM inhibited the proliferation of OA chondrocytes.

Effects of FCM on the metabolism of OA chondrocytes

Using the optimal chromatography condition described above, representative LC-MS total ion current (TIC) chromatograms from both groups in the positive and negative ion modes are shown in Fig. 2. The analytical stability of the system throughout the experiment was evaluated by analyzing QC samples. The PCA results (Fig. 3) show that the QC samples (red circle) were tightly clustered together, indicating good stability of the LC-MS system, which was sufficient for metabolomic analysis.

Metabolomic analysis

To determine whether FCM would likely alter the metabolic pattern of OA chondrocytes and whether the metabolite concentration would change significantly, multivariate data analysis techniques were conducted, including PCA, PLS-DA, and OPLS-DA, based on the LC-MS data, to explore the tendency of groups in both ESI+ mode (Fig. 4A) and ESI- mode (Fig. 4B). A clear separation in the PCA and PLS-DA score plots between control and model groups was noted, which indicated that they had different metabolic profiles. In addition, the total variable of principle components (R²Y) of the PLS-DA model in positive and negative modes was 0.993 and 0.999, and Q² was 0.954 and 0.955, respectively. These results indicated that the PLS-DA model exhibited goodness of fit and good prediction. Furthermore, to maximize the discrimination between the two groups, OPLS-DA analysis was carried out. OPLS-DA loading plots revealed that this model was efficient and showed clear separation between the FCM-treated group and the control group (R²X = 0.829, R²Y = 0.979, and Q² = 0.706). Moreover, a permutation test with 200 iterations confirmed that the constructed PLS-DA and OPLS-DA model was valid and not over-fitted.

Identification of significant metabolites

According to the OPLS-DA analysis, significant differences in metabolites between the FCM-treated group and the control group were identified. Using a VIP > 1.0 in the OPLS-DA models and $p < 0.05$ using a two-tailed Student's t-test, the metabolites with significant changes were screened out. There were 131 metabolites with VIP > 1.0 and $p < 0.05$ after FCM treatment (Fig. 5), of which 91 were mainly increased to various degrees, whereas the other 40 mainly showed a downward trend, indicating that FCM affected the metabolic secretion of substances in the OA chondrocytes and caused a significant change in their content. Fig. 6 shows the changes of amino acids with significant differences before and after coculturing. After coculturing with FCM for 48 h, we observed increased levels of 14 amino acids and declined levels of 3 amino acids in the OA chondrocytes culture medium.

Classification of metabolites between the OA + FCM 0 h group and OA + FCM 48 h group

Using the HMDB for classification (Fig.6B), of the 131 metabolites, 24% were sub-grouped as organic acids and derivatives, about 15% were amino acids (shown in Fig. 6A), peptides, and analogs, about 9% were lipids and lipid-like molecules, 8% were carbohydrates and carbohydrate conjugates, and 7% were pyrimidines and pyrimidine derivatives and purines and purine derivatives.

These metabolites were primarily located in the cytoplasm (53%), membrane (21%), extracellular (19%), and mitochondria (19%) cellular locations. In terms of biofunction distribution, these metabolites mainly participated in the biological processes of amino acid and energy metabolism and lipid metabolism, among others.

Effects of FCM on metabolic pathways of OA chondrocytes

Functional analysis showed that the differential metabolites identified are primarily involved in amino acid metabolism, energy metabolism, and lipid and oxidative stress metabolism. For 4 pathways in total, significantly different activity levels were discovered in pairwise comparisons. As illustrated in Fig. 7A, the analysis results showed that the main affected pathways were alanine, aspartate, and glutamate metabolism, the citrate cycle (TCA cycle), arginine and proline metabolism, and phenylalanine metabolism. Fig. 7B shows the correlation networks of the main potential affected pathways.

Discussion

It is evident that the IFP might interact with cartilage to participate in the development of OA, however, the underlying mechanism remains unclear. In this study, by coculturing FCM derived from human OA IFP and chondrocytes from OA patients, we found that FCM might inhibit the proliferation of OA chondrocytes. Furthermore, a total of 131 metabolites obtained in the culture media of OA chondrocytes were significantly changed. Pathway analysis revealed that those metabolic pathways significantly affected in OA chondrocytes included the TCA cycle, amino acid metabolism, and glutamine metabolism. These results suggest that IFP has a deleterious effect on cartilage, and the involvement of the IFP in the

development of OA might be associated with the effect of induced metabolic disturbances of chondrocytes.

Effect of FCM on TCA cycle pathways of human OA chondrocytes

The TCA cycle is a key biochemical pathway that provides cellular energy in the form of ATP by oxidative conversion of carbohydrates [14]. The increased levels of citric acid and succinic acid and decreased levels of isocitrate in the culture medium OA chondrocytes after treatment of FCM indicate enhanced activity of the TCA cycle, possibly due to perturbed metabolism in the cartilage cells. Akhbari et al.'s [15] study on synovial fluid (SF) showed that when compared with the metabolites of the SF in rheumatoid arthritis patients, OA patients had higher levels of metabolites involved in both TCA and glycolysis. Another study examined the metabolites of SFs between early- and late-stage OA and found that TCA intermediate metabolites including malate, citrate, succinate, and fumarate levels were significantly higher in the late-stage OA group than in the early-stage OA group, suggesting that the upregulation of the TCA pathway is greater in the late stage of OA [16]. In addition, a study on urine also indicated that the increased levels of TCA intermediate metabolites in the urine of inflammatory OA participants compared with non-OA controls suggested an enhanced activity of the TCA cycle [14]. Briefly, these data suggest that the TCA pathway is upregulated in OA, and our results agree with these studies, demonstrating that the upregulated TCA pathway of OA is most likely to increase the production of ATP needed to repair damaged cartilage. On the other hand, most of the enzymes involved in the TCA cycle are located inside the mitochondrial matrix of the cartilage cells, and the enhanced TCA cycle activity provides metabolic evidence of mitochondrial dysfunction of the cartilage cells in OA [17]. Back to our findings, these results showed that FCM treatment accelerated the injury of OA chondrocytes by enhancing the TCA cycle activity.

Effect of FCM on amino acid metabolism of human OA chondrocytes

Amino acids, small molecule metabolites, are considered the most important biological compounds, being involved in the synthesis of proteins and hormones and playing prominent metabolic and physiological roles (such as immune function, cytokine secretion) within organisms [18]. The current literature data suggest that the metabolic pathogenesis of OA might be significantly related to perturbations of amino acid metabolism [19]. In our study, after coculturing with FCM for 48 h, we observed increased levels of two main amino acids (threonine and glutamate) and declined levels of four main amino acids (cysteine, proline, arginine, and tyrosine) in the OA chondrocyte culture medium. Previous studies have found increased levels of glutamate and aspartate in dialysates from the injured joint of rats with anterior cruciate ligament transection (ACLT) [20]. Furthermore, the contents of glutamate and aspartate in the SF of patients with synovitis are 54 times and 28 times higher than those of healthy controls, respectively [21]. In addition, sheep with ACLT exhibited decreases in serum concentrations of tyrosine, valine, and isoleucine at week 4 after surgery, compared with the sham control [22]. Moreover, a study evaluated the short-term metabolic response of human chondrocytes to mechanical loading and suggested that rates of serine and threonine metabolism were increased. In

rabbits with an ACLT injury, there were negative correlations between histological assessments and changes in plasma concentrations of arginine [23]. These findings indicate that the decrease of arginine plasma concentration was due to the process and progress of OA. In summary, the increase of glutamate and threonine levels and the decrease of tyrosine and arginine levels could induce the occurrence and development of OA. Furthermore, prolines are thought to inhibit cell oxidative damage and have anti-inflammatory effects [24]. In our study, after coculturing with FCM, the levels of arginine, proline, and tyrosine decreased, and the levels of glutamate and threonine increased in the culture medium of OA chondrocytes, indicating that FCM promotes the inflammatory response of OA chondrocytes and IFP has a deleterious effect on cartilage.

Effect of FCM on glutamine metabolism of human OA chondrocytes

Glutamine is a key source of carbon secondary only to glucose for energy production and anabolic processes [25]. Recently, a study found that glutamine suppressed OA progression [26]. Glutamine is a precursor of glucosamine and can upregulate glucosamine synthesis in chondrocytes, and glucosamine could inhibit cartilage destruction and promote biosynthetic activity of chondrocytes [27]. A study observed that the concentration of glutamine was lower in the SF from OA patients than in normal SF [15]. In addition, 2-keto-glutaramic acid (a deaminated metabolite of glutamine under abnormal conditions) has been found to be increased in the urine of inflammatory OA participants, indicating that glutamine metabolism might be disturbed in the chondrocytes [28]. Furthermore, Wang et al. [29] indicated that growth plate (GP) and articular cartilage chondrocytes exhibited metabolic plasticity that allowed these cells to switch to glutamine oxidation to support energy production when glucose metabolism was compromised. Glutamine oxidation enables GP chondrocytes to survive in glucose-limited conditions, however, normal proliferation and matrix production by these cells is impaired. In a word, in OA progression, glutamine metabolism could be disturbed in the chondrocytes, and lower glutamine might indicate the cartilage destruction of the joint. Our observations suggested that after coculturing with FCM, the level of glutamine declined. This result supports that IFP has a deleterious effect on cartilage. However, the mechanisms of glutamine involvement in OA pathogenesis are unclear. During stress loading, exogenous glutamine supplementation has been shown to increase the concentration of glutathione (an essential intracellular antioxidant) that protects cells from injury. Moreover, glutamine has been shown to be vital for fueling mitochondrial metabolism [30], and the IFT might disrupt glutamine metabolism, resulting in mitochondrial dysfunction, and finally lead to the development of OA. Further research is needed to determine the mechanisms of the crosstalk between the IFP and cartilage.

There are a couple of potential limitations of the current study that must be acknowledged. First, we only evaluated the changes to OA chondrocyte culture medium metabolites after FCM treatment. The underlying mechanism of the interaction between cartilage and IFP could be better understood together with the intracellular metabolites, which should be evaluated in the future. Furthermore, our results indicated that IFP could disturb the TCA cycle, amino acid metabolism, and glutamine metabolism of OA chondrocytes, however, further investigations are warranted to verify the results of metabolomics.

Conclusion

In conclusion, we have shown that IFP has a deleterious effect on cartilage, and IFP involvement in the development of OA could be associated with disturbances to the chondrocyte TCA cycle, amino acid metabolism, and glutamine metabolism, among others. These metabolic disturbances could provide insight into the mechanisms underlying OA, and beyond that, to develop new strategies to slow down the degenerative process and explore an effective and timely diagnosis of the disease.

Abbreviations

FCM: fat conditioned media

OA: osteoarthritis

LC-MS: liquid chromatography and mass spectrometry

PCA: principal component analysis

PLS-DA: partial least squares discriminant analysis

OPLS-DA: orthogonal projection to latent structures-discriminant analysis

IFP: infrapatellar fat pad

MMP: matrix metalloproteinase

QC: quality control

UHPLC/TOF-MS: liquid chromatography-quadrupole/time of flight mass spectrometry.

ACLT: anterior cruciate ligament transection

GP: growth plate

Declarations

Acknowledgments

None

Author contributions

All authors read and approved the final manuscript. NZ and GXN conceived research concept, NZ and ZPY designed and performed the experiments, analyzed data, and prepared figures, LT, JTL and YC were

responsible for sample collection and sample storage, and GXN drafted, revised and edited the manuscript.

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Availability of data and material

Not applicable.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

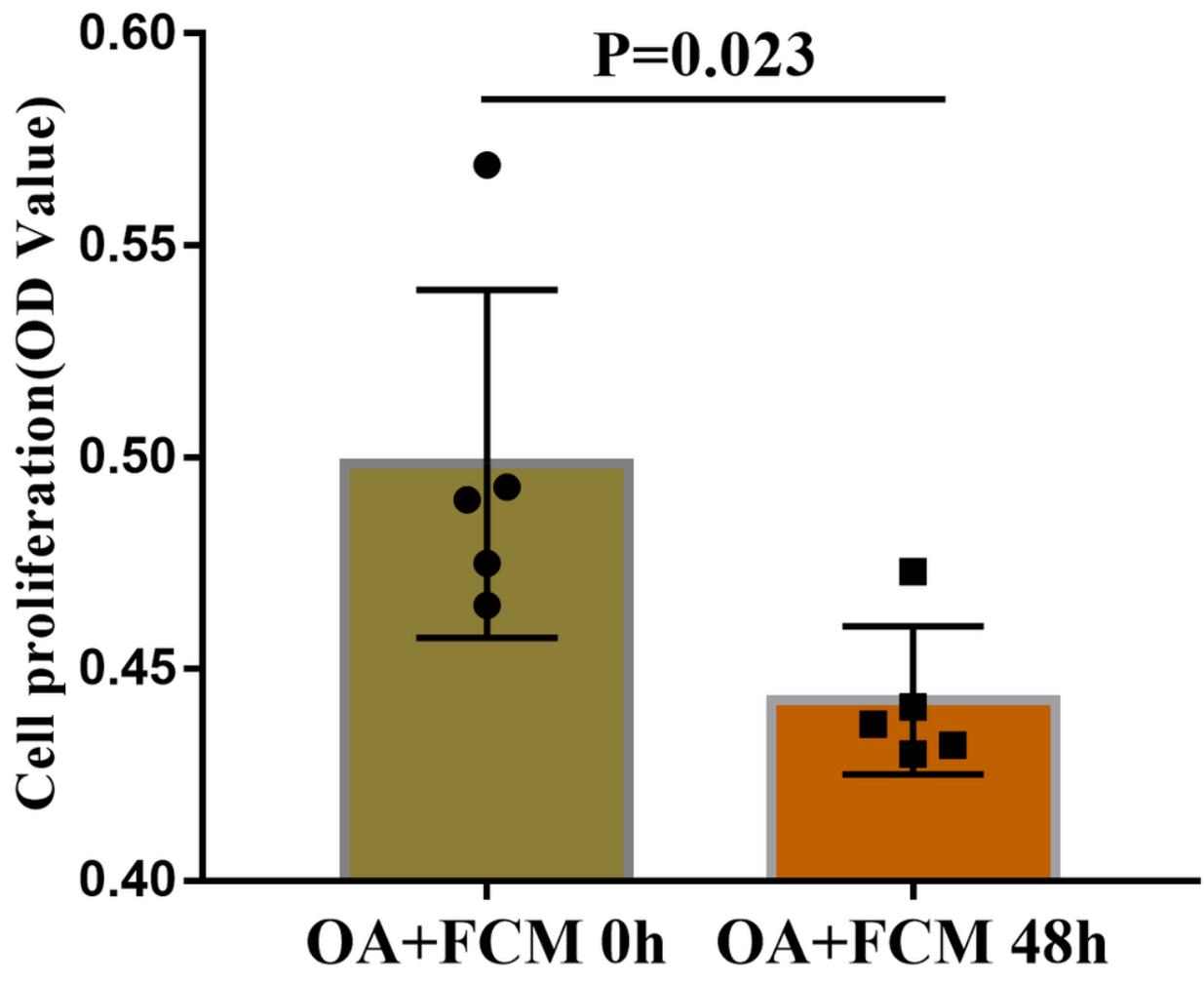


Figure 1

The proliferation of OA chondrocytes under the stimulus of FCM.

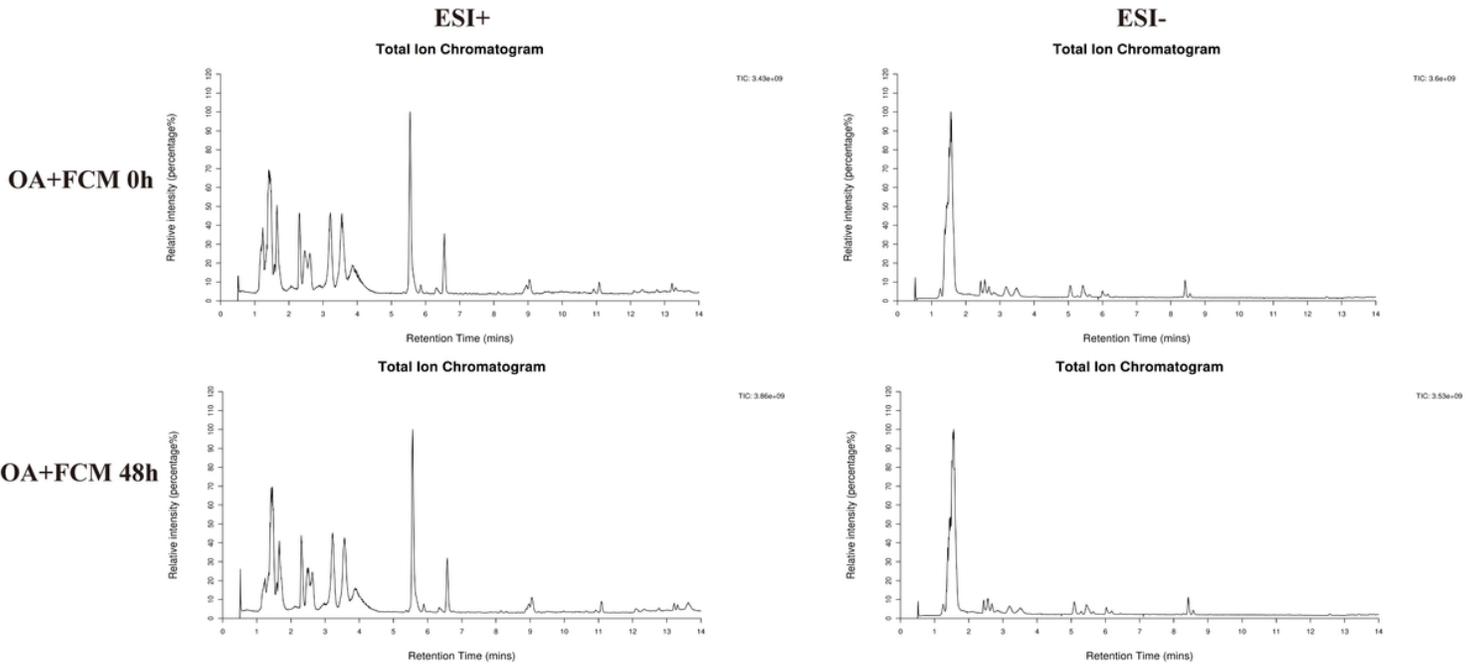


Figure 2

The total ion chromatograph of the OA + FCM 0 h and OA + FCM 48 h groups in both positive and negative (ESI+ and ESI-) modes.

Figure 3

The PCA scores plot-obtained QC samples (red circle) and experimental samples (green circle). The QC samples were tightly clustered together, indicating good stability of the LC-MS system

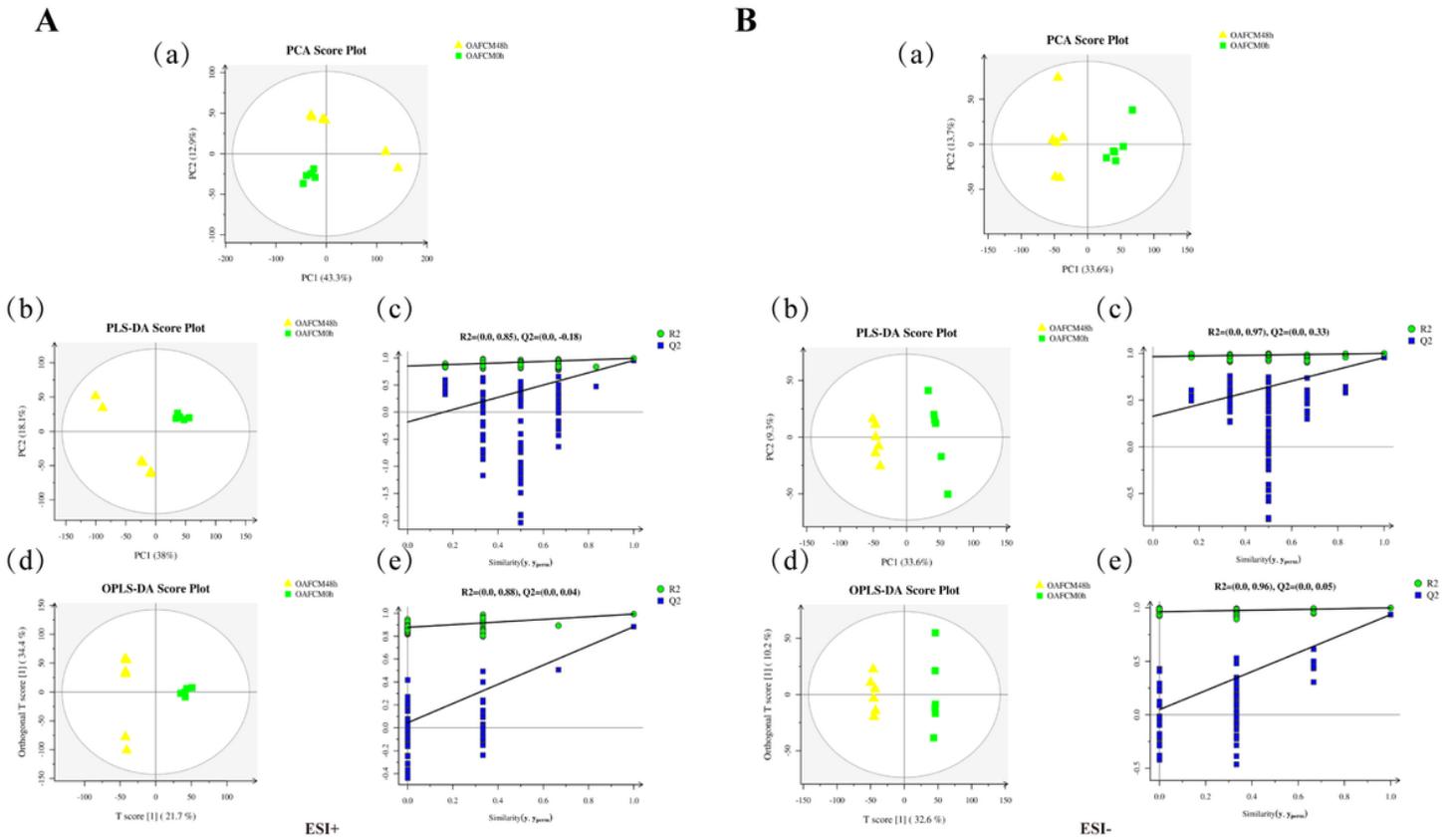


Figure 4

Multivariate analysis for differential profiles among the OA + FCM 0 h and OA + FCM 48 h groups. A: ESI+: (a) PCA scores plot, (b) PLS-DA scores plot, (c) PLS-DA cross-validation plot (d) OPLS-DA (e) OPLS-DA cross-validation plot. B: ESI- : (a) PCA scores plot, (b) PLS-DA scores plot, (c) PLS-DA cross-validation plot (d) OPLS-DA (e) OPLS-DA cross-validation plot

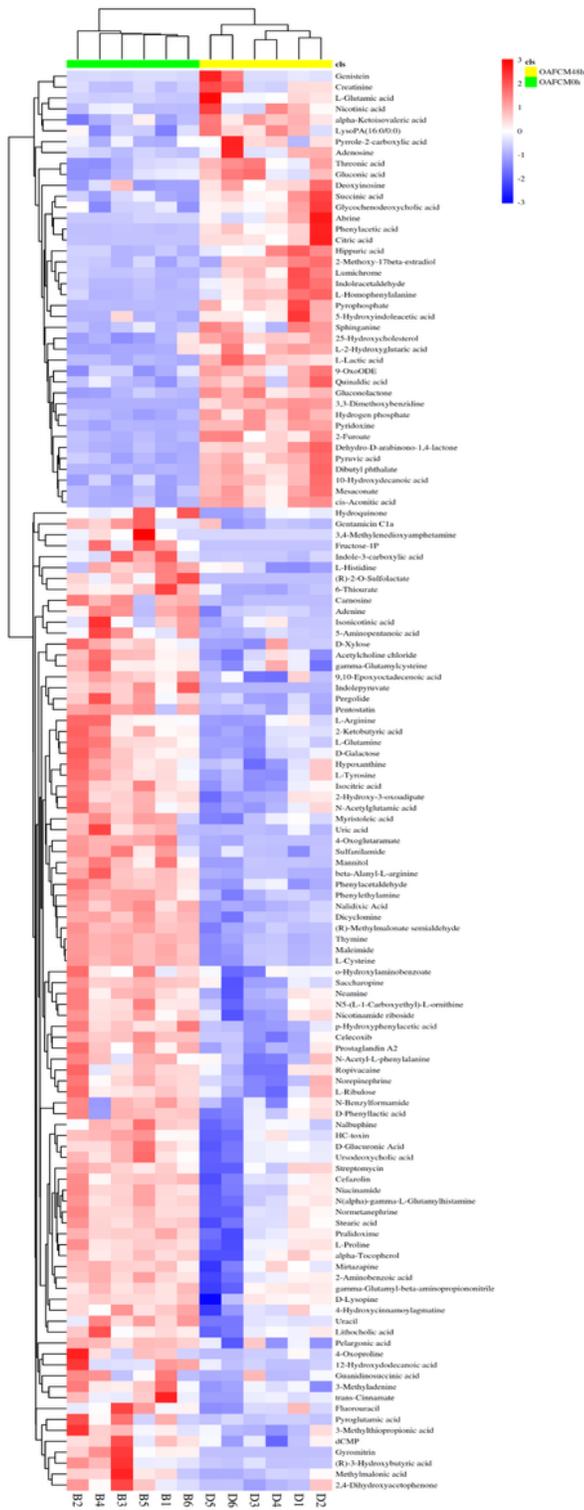


Figure 5

Heat map of the significantly changed metabolites between the OA + FCM 0 h and OA + FCM 48 h groups.

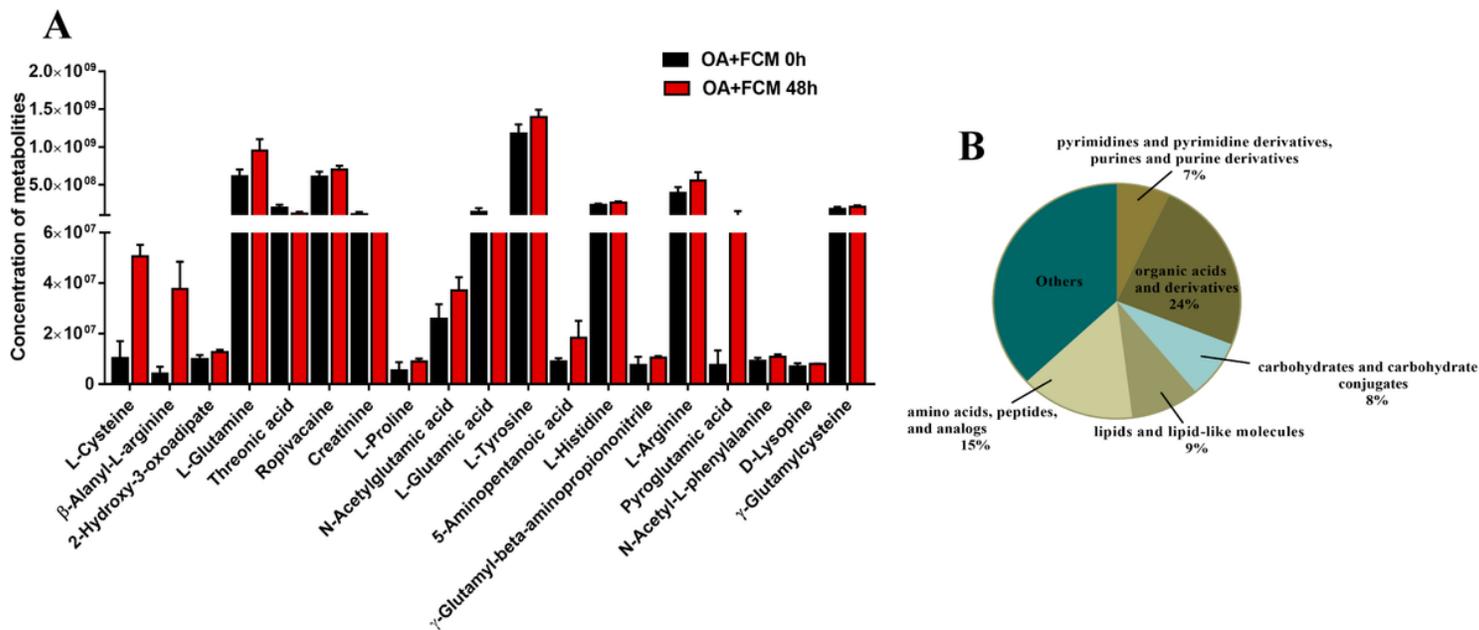


Figure 6

Classification of metabolites between the OA + FCM 0 h group and OA + FCM 48 h group. A: Quantitative expression of the significantly changed amino acids between the OA + FCM 0 h and OA + FCM 48 h groups. B: The classification of significant metabolites between the OA + FCM 0 h group and OA + FCM 48 h group.

Figure 7

Summary of pathway analysis. A: Each point represents one metabolic pathway, the size of the dot and shades of color are positively correlated with the impact of the metabolic pathway. B: Mapping the correlation networks of main potential affected pathways of OA chondrocytes by FCM. The red indicates a significantly upregulated metabolite, and green indicates downregulated. (TCA cycle: tricarboxylic acid cycle, ROS: reactive oxygen species).