Simvastatin Retards Cartilage Degradation and Improves Subchondral Bone Microstructure in Osteoarthritis Mice Induced by High-fat Diet

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Abstract

Objectives

This study aimed to evaluate the effects of simvastatin on the metabolism of cartilage and subchondral bone in a mice model of osteoarthritis (OA) induced by obesity.

Methods

We randomly assigned thirty C57BL/6J mice into 3 groups: the Control group received normal diet, the HFD group and HFD+S group received high-fat diet, HFD+S group were simultaneously treated with simvastatin (10 mg/kg/day) for 8 weeks. The pathology of OA was assessed by histomorphology analyses, immunohistochemistry, micro-computed tomography and enzyme-linked immunosorbent assay.

Results

Histomorphological analysis revealed that OA was significantly exacerbated by the HFD-induced obesity and markedly alleviated by the simvastatin intervention. In details, simvastatin ameliorated the abnormal metabolic status and cartilage lesions, significantly increased aggrecan and collagen-II expression and decreased the expression of MMP-13. Furthermore, the results of micro-computed tomography analysis revealed that the HFD+S group exhibited higher BMD, BV/TV, and Tb.N values but a lower Tb.Sp value than that of the HFD group. Serum COMP concentrations, the number of adipocytes in subchondral bone marrow and the number of osteoclasts on trabecular bone surface were significantly correlated with OARSI score.

Conclusions

In conclusion, HFD-induced obesity aggravates articular degeneration and abnormal metabolic pathology in subchondral bone, which could be reversed by the intervention of simvastatin, suggesting that simvastatin may be a potential candidate for amelioration of the progression of OA.

Introduction

Obesity is now one of the most urgent global healthcare challenges. Obesity reduces both life expectancy and quality of life[5], and is closely associated with the risk of developing metabolic, cardiovascular, muscle skeletal and many other diseases[7]. A significant contributor to obesity-induced disability is osteoarthritis (OA)[9], which is a worldwide public health problem that causes long-term pain and disability[19], and its disability-adjusted life years index rose by 34.8% between 2005 and 2015[3].

OA is a whole-joint disorder involving the cartilage, subchondral bone, synovium, ligament and joint capsule. Many pathologies are related to OA, including cartilage degeneration, subchondral bone loss, and synovial inflammation. However, the potential mechanism of obesity-related OA is complex and

controversial[36]. One contributing factor appears to be overweight-associated mechanical overload, which has long been pointed out to explain the link between obesity and osteoarthritis. Related clinical studies also showed positive correlations between BMI and the onset or progression of knee osteoarthritis[26]. In addition to the abnormal loading, obesity-related systemic metabolic factors may play an important role in the pathological process of OA. Various regular cellular processes are disturbed by systemic metabolic diseases, such as mitochondrial bioenergetics, nutrient sensing, and glycolysis. Consistently, damaged cellular metabolism is the most important characteristics of chondrocytes in OA[6].

Current medical treatment strategies for OA are focused on pain relief and symptom control rather than disease improvement. Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitor, has been widely used in the initial treatment of hyperlipidemia and prevention of cardiovascular disease. Furthermore, simvastatin has been reported to have a protective effect on the cartilage in the knee joint, such as promoting the proliferation and inhibiting the apoptosis of chondrocytes[39], stimulating bone formation[10], and enhancing anti-inflammatory effects[30]. Previous in vivo studies have shown that application of statins reduces cartilage degradation in rabbits with experimental osteoarthritis and inhibits the expression of matrix metalloproteinase-3 (MMP-3) in a mechanically induced knee osteoarthritis[2, 12]. Moreover, in vitro experiments have shown that statins up-regulate the mRNA levels of bone morphogenetic protein-2 (BMP-2), aggrecan, collagen-II and increase the synthesis of proteoglycan in rat chondrocytes[15]. These findings suggest a possible effect of simvastatin for treating OA involving cartilage degeneration and subchondral bone loss.

The purpose of this study is to further explore the pathogenesis of OA induced by obesity in high-fat diet (HFD) mice and to investigate the therapeutic effects of simvastatin on knee OA.

Methods

Animals and treatments

All experimental procedures were approved by the Institutional Animal Care and Use Committee. Thirty C57BL/6J mice (age: 3 months old; Vital River Experimental Animal Technical Co., Ltd., China) were used in this research. All mice were randomized into three groups (n = 10/group): mice with normal diet + vehicle (distilled water) (Control), mice with High-fat diet + vehicle (HFD), mice with HFD + Simvastatin (HFD+S). Drug administration was initiated at the same day the HFD was given. Mice in the HFD+S group received oral gavage of Simvastatin (SL Pharmaceutical Co., Ltd. Beijing, China) at a dosage of 10 mg/kg/day, other groups were given distilled water. After 8 weeks of treatment, all mice were euthanized to collect blood and samples. For details see Supplementary Methods.

Macroscopic observation

After all mice were euthanized, the abdominal subcutaneous fat was photographed with a Canon 550D digital camera (Canon, Tokyo, Japan).

Body weight, Waist circumference and Body mass index (BMI)

Body weight was measured weekly and waist circumference was measured before the macroscopic observation. Body mass index (BMI) was calculated using the formula: (Weight in kg/Body length in m^2) [18].

Histological assessments

In the mice, perirenal adipose tissue and knee joints were harvested and sequentially fixed in 10% neutral-buffered formalin, dehydrated in a graded series of ethanols and embedded in paraffin wax (bone tissue needs decalcification), and finally cut into 5-μm sections. Adipose tissues were stained with hematoxylin and eosin (H&E) to observe the surface area of fat cells. Bone tissues sections were stained for light microscopy with H&E to evaluate the pathological changes in subchondral bone marrow fat cells, cartilage degradation was assessed using Safranin-O and fast green staining and scored according to the Osteoarthritis Research Society International (OARSI) scoring system (Supplementary Table 1). We also performed tartrate-resistant acid phosphatase (TRAP) staining to evaluate $TRAP^+$ osteoclasts within the subchondral bone following a standard protocol (CK20203, MultiSciences Co., Ltd., Zhejiang, China). All histological evaluation was performed by 2 independent researchers in a blinded manner.

Immunohistochemistry

The expression levels of aggrecan (1: 100; Boster Co., Ltd., Wuhan, China), collagen-II (1: 100; Boster Co., Ltd., Wuhan, China), ADAMTS-4 (1: 100; Boster Co., Ltd., Wuhan, China), MMP-13 (1: 100; Boster Co., Ltd., Wuhan, China), caspase-3 (1: 100; Boster Co., Ltd., Wuhan, China) in cartilage and the expression of Osteocalcin (OCN) (1: 100; Boster Co., Ltd., Wuhan, China) in subchondral bone were analyzed. For details see Supplementary Methods.

Micro-computed tomography (micro-CT) analysis

To investigate the changes in the subchondral bone micro-architecture, the knee joint was imaged using a Micro-CT (SkyScan1176 Software: Version1.1 (build 6), Bruker, Kontich, Belgium). Bone mineral density (BMD, g/cm^3), bone volume/tissue volume (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, mm), and structure model index (SMI) were calculated.

Biomarker assays

Serum concentrations of cartilage oligomer protein (COMP) (CUSABIO Co., Ltd., Wuhan, China), CTX-II (Abbexa Co., Ltd., Cambridge, UK) and IL-6 (Abbexa Co., Ltd., Cambridge, UK) were determined using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. The data were measured using an iMARK Reader (BioRad Laboratories Inc., USA).

Data analysis and statistics

All data are presented as mean ± standard deviation or mean with 95% confidence interval and were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Non-Gaussian distributed data were compared using Kruskal-Wallis and Mann-Whitney non-parametric analysis. One-way analysis of variance (ANOVA) followed by Tukey HSD or Dunnett's T3 test were used for pairwise comparison of data with a Gaussian distribution and homogeneity of variance. Spearman's rank correlation analyses were used to investigate correlations between the concentration of serum COMP, CTX-II, IL-6, the number of adipocytes in subchondral bone marrow, the number of osteoclasts on trabecular bone surface and OARSI score. The level of statistical significance was established at $P < 0.05$.

Results

Macroscopic findings of abdominal subcutaneous fat

In HFD-treated mice, macroscopic images showed evident abnormal accumulation of subcutaneous fat in the abdomen, while simvastatin treatment reversed the abnormality in the HFD+S group (Figure 1A).

Changes in body weight, waist circumference and BMI index

The weight of HFD mice was significantly higher than that of the Control group from the second week ($P < 0.05$). Simvastatin down-regulates this abnormal weight gain, and the weight of HFD+S group increased only slightly compared to Control group. At the end, the body weight of HFD mice was significantly higher than Control mice ($P < 0.05$). The intervention of simvastatin significantly reduced the weight of mice fed a HFD ($P < 0.05$) (Figure 1B). The waist circumference and BMI index of mice in HFD group substantially increased compared with the Control group at 8 weeks ($P < 0.001$ and $P < 0.01$). Compared with HFD mice, the waist circumference and BMI index of HFD+S mice was significantly reduced after treatment ($P < 0.001$, and $P < 0.05$) (Figure 1C, E). There was no statistical difference in the body length of mice in each group (Figure 1D).

Histological evaluation of adipose tissue

The mean surface area of adipocytes observed in the HFD group was significantly increased compared to the control group ($P < 0.001$), whereas after simvastatin treatment, mice in the HFD+S group showed a significantly lower area than that in the HFD group ($P < 0.001$) (Figure 2A, B). The high-fat diet greatly promoted the differentiation of adipocytes in the subchondral bone marrow of mice (Figure 2C). The number of adipocytes in subchondral bone marrow was significantly higher in the HFD group than in the Control group ($P < 0.001$). The effects were reversed by gavage of simvastatin. However, there was still significant difference between HFD+S group and Control group ($P < 0.001$) (Figure 2D).

Histological changes in articular cartilage

In the Control group, the cartilage surface and the structures of chondrocytes were normal, Safranin-O staining of extracellular matrix (ECM) was evenly distributed. With the induction of high-fat diet, the articular cartilage of HFD group exhibited extensive cartilage lesions, and Safranin-O staining area was

significantly reduced. The administration of simvastatin alleviated the severity of cartilage degeneration (Figure 2E). The HFD group presented a significantly higher OARSI score than the Control group ($P < 0.001$), and the score was significantly decreased after simvastatin intervention ($P < 0.001$) (Figure 2F).

Simvastatin improve cartilage metabolism

Aggrecan and collagen-II expression was significantly lower in the HFD group than in the Control group ($P < 0.001$), whereas these two proteins expression was significantly higher in the HFD+S group than in the HFD group ($P < 0.001$). (Figure 3A-D). Significantly higher MMP-13 and caspase-3 expression level was observed in the HFD group than in the Control group ($P < 0.001$ and $P < 0.01$). After simvastatin intervention, the expression level of MMP-13 but caspase-3 in the HFD+S group was significantly reduced ($P < 0.001$) (Figure 3G-J). There was no significant difference in the expression level of ADAMTS-4 among the groups. (Figure 3E, F).

Simvastatin improve subchondral bone metabolism

OCN immunohistochemical staining and TRAP staining showed a significantly increased number of osteoblasts and osteoclasts on trabecular bone surface in the HFD mice compared with Control mice ($P < 0.001$). However, the intervention of simvastatin significantly decreased the number of osteoblasts and osteoclasts in HFD+S group ($P < 0.01$ and $P < 0.001$) (Figure 4)

Micro-CT parameters of subchondral bone

The Micro-CT image of the femur and tibial subchondral bone of mice in each group are shown in Figure 5A. Quantitative analysis of the subchondral bone of the tibia showed that HFD mice exhibited significantly lower BMD, BV/TV, Tb.N, Tb.Th, and significantly higher Tb.Sp than that in Control mice ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). Compared with those of the HFD group, the BMD, BV/TV, and Tb.N values of the HFD+S group were significantly higher, but the Tb.Sp value was significantly lower ($P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively) (Figure 5B-G).

Biomarker analysis

The concentration of serum COMP and IL-6 was significantly higher in the HFD group than in the Control group ($P < 0.001$ and $P < 0.05$, respectively), after the intervention of simvastatin, the COMP and IL-6 concentrations of mice in the HFD+S group decreased, but there was no significant difference compared with the HFD group (Figure 6A, C). The serum CTX-II concentration was not significantly different among groups (Figure 6B).

Correlation analysis

Significant positive correlation was observed between Serum concentrations of COMP (Figure 6D), the number of adipocytes in subchondral bone marrow (Figure 6E), the number of osteoclasts on trabecular bone surface (Figure 6F) and OARSI score ($r = 0.471$, $P < 0.01$, $r = 0.892$, $P < 0.001$, and $r = 0.838$, $P <$

0.001, respectively). However, there were no significant correlation between serum concentration of CTX-II, IL-6 and OARSI score (r = 0.221, P = 0.242, and r = 0.227, P = 0.228, respectively) (Supplementary figure 1A, B).

Discussion

The results of the present study demonstrate that HFD-induced obesity aggravated the degradation of cartilage matrix and the destruction of subchondral bone in mice. All these degenerative changes in the OA model were retarded by simvastatin inhibiting overactivated subchondral bone remodeling and cartilage catabolic metabolism.

OA etiology studies generally suggested cartilage lesions are major pathologic changes of OA joints[13, 20]. The extracellular matrix (ECM) homeostasis imbalanced in OA cartilage[24], high-magnitude mechanical stress and high levels of inflammatory cytokines, which all exacerbate the inflammatory responses of OA[37]. In our study, the HFD group displayed distinct cartilage degeneration and a higher histological score than in the Control group. In order to further explore the underlying mechanism of cartilage impairment in obese mice, we detected the proteins related to cartilage metabolism and chondrocyte apoptosis. Aggrecan and collagen-II are components of chondrocyte ECM, which plays an important role in maintaining the proper function of cartilage[14, 27]. MMP-13 and ADAMTS-4, two matrix-degrading enzymes, involved in the process of degrading aggrecan and collagen-II and usually highly expressed in OA cartilage[28, 34], and the expression of caspase-3 reflects the degree of apoptosis of chondrocytes[38]. Immunohistochemical results indicated that the expression levels of MMP-13 and caspase-3 were significantly increased, whereas the expression of aggrecan and collagen-II was significantly decreased in HFD mice. These findings indicate that HFD-induced obesity leads to enhanced catabolism of cartilage and apoptosis of chondrocytes, which may be the primary cause of cartilage impairment in the model used in the present study.

Previous studies have pointed out that in OA, subchondral bone undergoes an abnormal remodeling process with the regulation of both mechanical and biological signals[17]. The overactive osteoblast metabolism might be responsible for the aberrant mineralization of subchondral bone in OA[25]. In our study, OCN immunohistochemical staining showed a significantly higher number of osteoblasts on trabecular bone surface in the HFD mice compared with Control mice. Besides, TRAP staining indicates a significant increase the osteoclast number in obesity-induced OA. This elevated number and distribution of osteoclasts adjacent to the trabecular bone is also considered a representative phenomenon in subchondral bone marrow lesions[35].

Micro-CT analysis of the subchondral bone microstructure is another way to assess the level of detail regarding the pathological changes present in subchondral bone[29]. previously showed that the abnormalities of subchondral bone were present in OA with high bone turnover and subsequent loss of bone mass[8]. In our study, the BMD, BV/TV, Tb.N and Tb.Th values of the subchondral bone in HFD mice were significantly reduced, but the Tb.Sp value was significantly increased, indicating that the

subchondral bone of obesity-induced OA mice was abnormal bone remodeling. Deleterious alterations of subchondral bone may transmit abnormal stress to articular cartilage and cause the deterioration of cartilage[23]. These findings suggest that subchondral bone plays a critical role in the progression of OA.

Statins are clinically used for the treatment of dyslipidemia currently. With the deepening of research, it has been discovered that statins, in addition to reducing blood lipids, also have therapeutic effects on many other diseases. There have been studies discussing the treatment of osteoarthritis with statins, which is found to be able to promote the proliferation of chondrocytes in the knee joint and inhibit their apoptosis, as well as delay the degradation of cartilage matrix[4, 22]. These reports revealed the therapeutic effect of OA after simvastatin administration. In this study, we confirmed that simvastatin had a protective effect on the impaired OA cartilage and subchondral bone.

From the data, the intervention of simvastatin significantly reduced the body weight, the accumulation of neutral lipid in visceral adipose tissue and the subchondral bone marrow adipocytes of mice with HFD. The weight loss effect of simvastatin has been confirmed in clinical studies[31]. Although it is not a weight loss drug in essence, simvastatin has a beneficial effect on the regulation of abnormal lipid metabolism to retard the progression of OA induced by obesity. Then, we found that simvastatin exhibits a significant cartilage-protective effect, which have been reported in previous in vitro studies[32] and in vivo studies[2] of knee OA. Our research showed similar results, simvastatin therapy significantly decreased the expression levels of MMP-13 in the OA cartilage of obesity mice and significantly increased the expression of aggrecan and collagen-II. Not only cartilage metabolism, the subchondral bone is also closely associated with the development and progression of OA[11]. In the present study, the deterioration of subchondral bone micro-architecture and abnormal metabolism was remarkably ameliorated by treatment with simvastatin: BMD, BV/TV, and Tb.N values significantly increased, and Tb.Sp values significantly decreased. These results demonstrate the inhibition of subchondral bone degeneration also exerts indirect protective effects on cartilage by avoiding secondary damage caused by abnormal mechanical stress and metabolism.

COMP is an important component of the cartilage ECM[1]. Many studies have shown that serum COMP is a potential diagnostic marker for the occurrence and progression of OA[16, 33]. In our study, we found that the serum COMP concentration in the HFD group was significantly higher than that in the Control group. After administration of simvastatin, the serum COMP concentration of obesity mice was reduced, although there was no statistical difference. In addition, the serum IL-6 levels of mice in the HFD group were significantly increased. As a systemic inflammatory factor, the elevated level of IL-6 could facilitate inflammatory response and further aggravate the degeneration of the knee joint[21]. Moreover, serum COMP concentration, the number of adipocytes in subchondral bone marrow and the number of osteoclasts on trabecular bone surface were significantly correlated with OARSI score. Thus, we believed that abnormalities in the subchondral microenvironment such as overactive adipogenic differentiation of bone marrow mesenchymal cells instead of osteogenic differentiation, may be another cause of exacerbate joint damage.

Conclusion

In summary, obesity aggravates articular degeneration, characterized by enhanced cartilage catabolism and the abnormal metabolic pathology and remodeling of subchondral bone. Simvastatin have a potential therapeutic effect on obesity-induced OA. For OA patient with obesity, simvastatin may be used to treat hyperlipidemia or weight control and also have protective effect on the knee joint. But for OA patient with no obesity, the side effects of simvastatin should also be noted. Further research is needed before it can be put into clinical practice.

Abbreviations

OA: osteoarthritis; HFD: High-fat diet; ECM: extracellular matrix

Declarations

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Not applicable

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Authors' contributions

LHT and GY conceived and devised the study. LHT, GY, LQQ and HYP performed the study. LHT, LQQ and HYP analyzed the data. LHT and TFM wrote the paper. TFM and ZL revised the manuscript. ZL obtained the funding and supervised the whole project. All authors have contributed to the final version and approved the publication of the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study design, procedures, and informed consent procedure were approved by the North China University of Science and Technology.

Competing interests

The authors declare that they have no competing interests.

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Figures

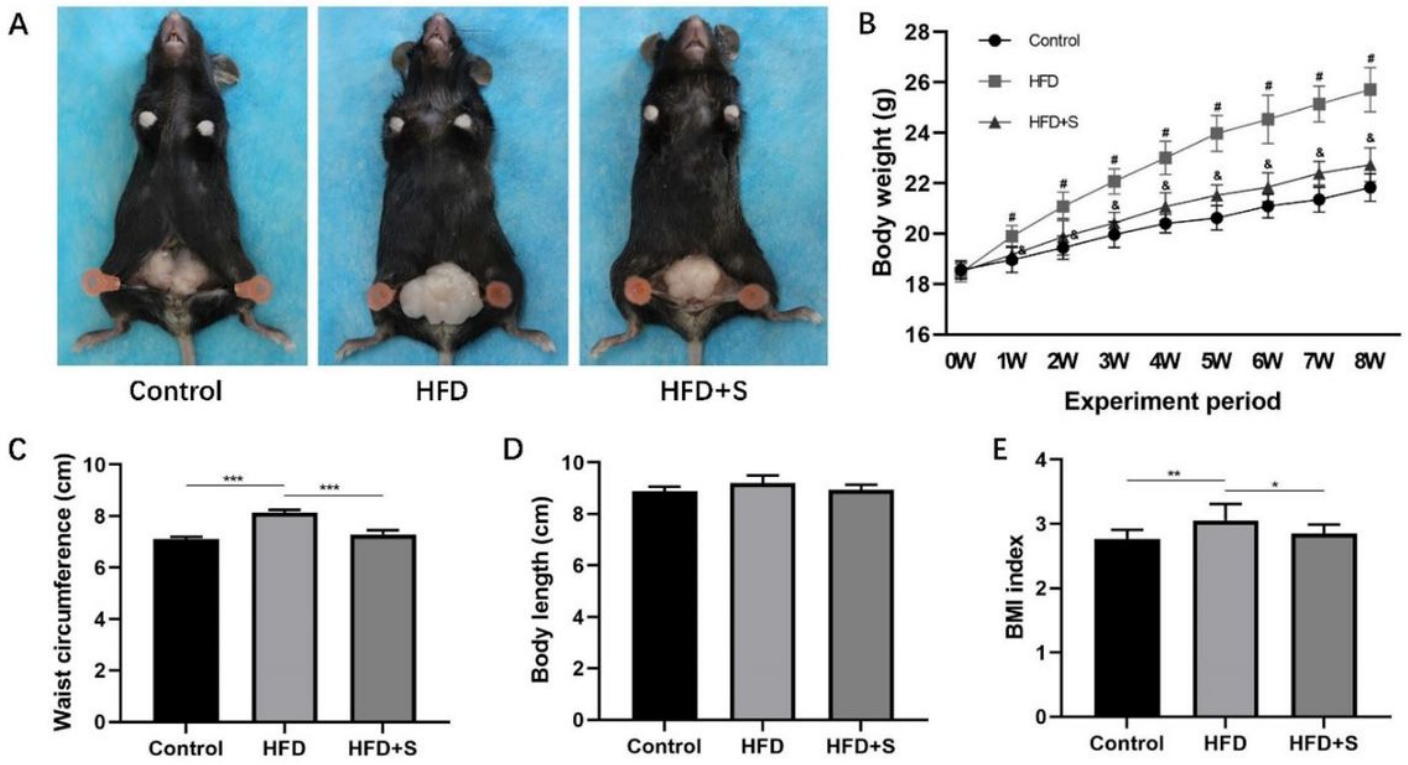

Figure 1

(A) The macroscopic images of abdominal subcutaneous fat and (B) body weight, (C) waist circumference, (D) body length, (E) BMI index in all studied groups. Data are expressed as mean ± standard deviation. #P < 0.05 vs Control group; &P < 0.05 vs HFD group; *P<0.05; **P<0.01; ***P<0.001.

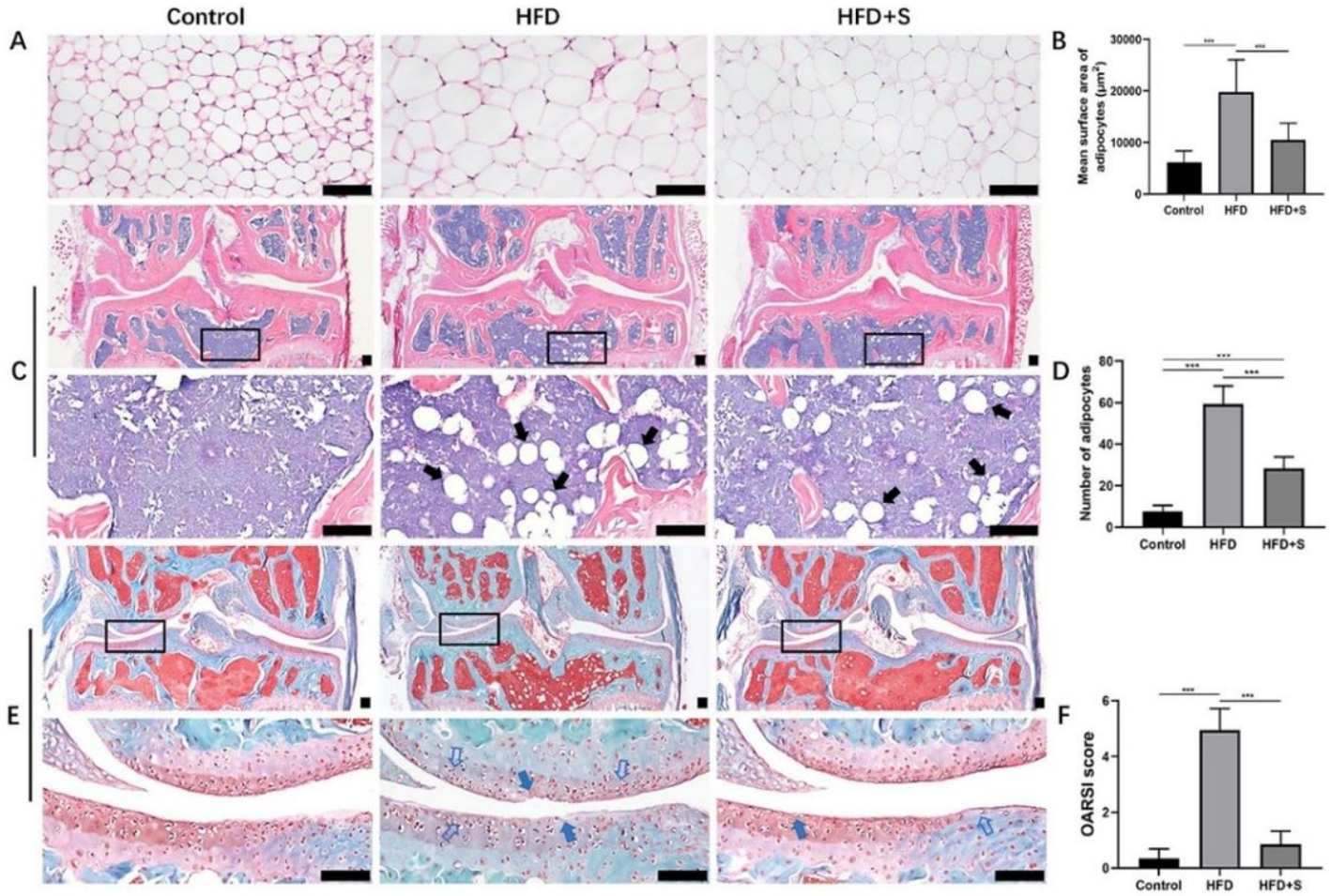

Figure 2

(A, C, E) Representative histological sections indicated that the accumulation of neutral lipid and the progression of OA was attenuated by simvastatin. (B) The quantified mean surface area of adipocytes among the groups. (D) The quantified number of adipose cells among the groups. (F) OARSI scores of each group. Black arrow indicates abnormal differentiation of adipocytes in the subchondral bone. Blank arrow indicates reduced Safranin-O staining. Blue arrow shows surface fissures. Data are expressed as mean with 95% confidence interval. Bar = 100 μm. *P<0.05; **P<0.01; ***P<0.001.

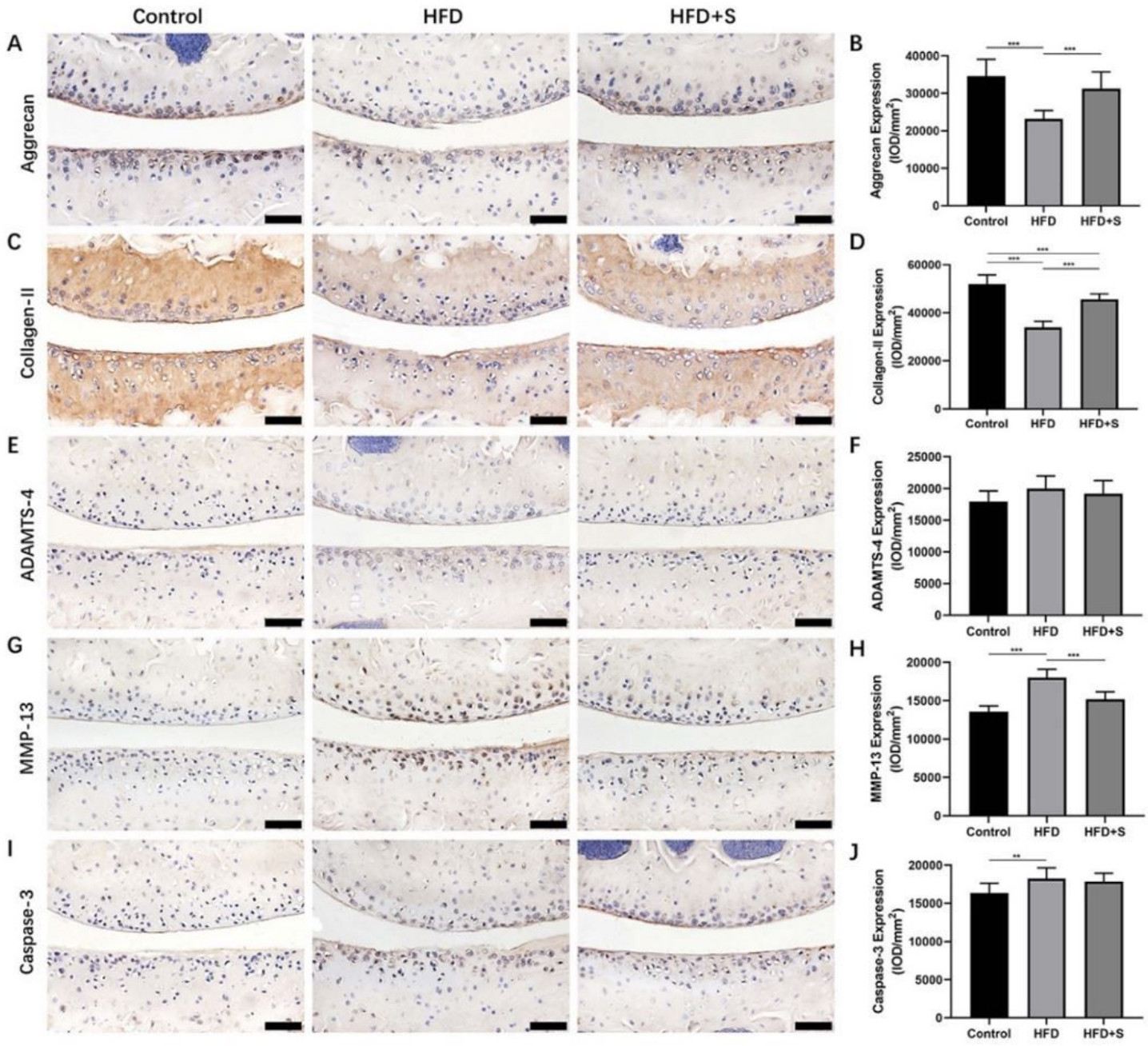

Figure 3

Immunohistochemical staining for aggrecan, collagen-II, ADAMTS-4, MMP-13 and caspase-3 in cartilage. (A, C, E, G, I) The intervention of simvastatin promotes aggrecan, collagen-II expression and inhibits MMP-13 expression in the cartilage of mice with a HFD. The level of ADAMTS-4 was not significantly different among the groups. (B, D, F, H, J) The quantified protein levels of aggrecan, collagen-II, ADAMTS-4, MMP-13 and caspase-3 in cartilage. Data are expressed as mean ± standard deviation. Bar = 50 μm. *P<0.05; **P<0.01; ***P<0.001.

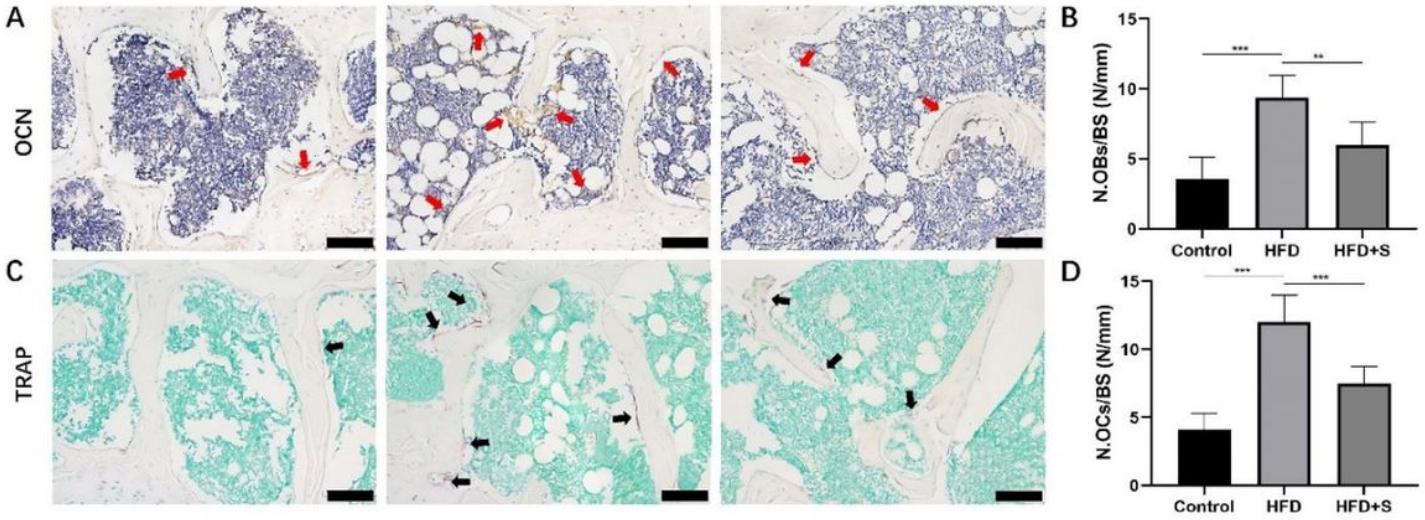

Figure 4

The intervention of simvastatin reverses the imbalanced subchondral bone metabolism in HFD mice. (A) Representative OCN-stained sections with quantification of the (B) number of osteoblasts (N. OBs) on trabecular bone surface (BS) in tibia subchondral bone. (C) Representative TRAP-stained sections with quantitation of the (D) number of osteoclasts (N. OCs). Data are expressed as mean with 95% confidence interval. Bar = 100 μm. *P<0.05; **P<0.01; ***P<0.001.

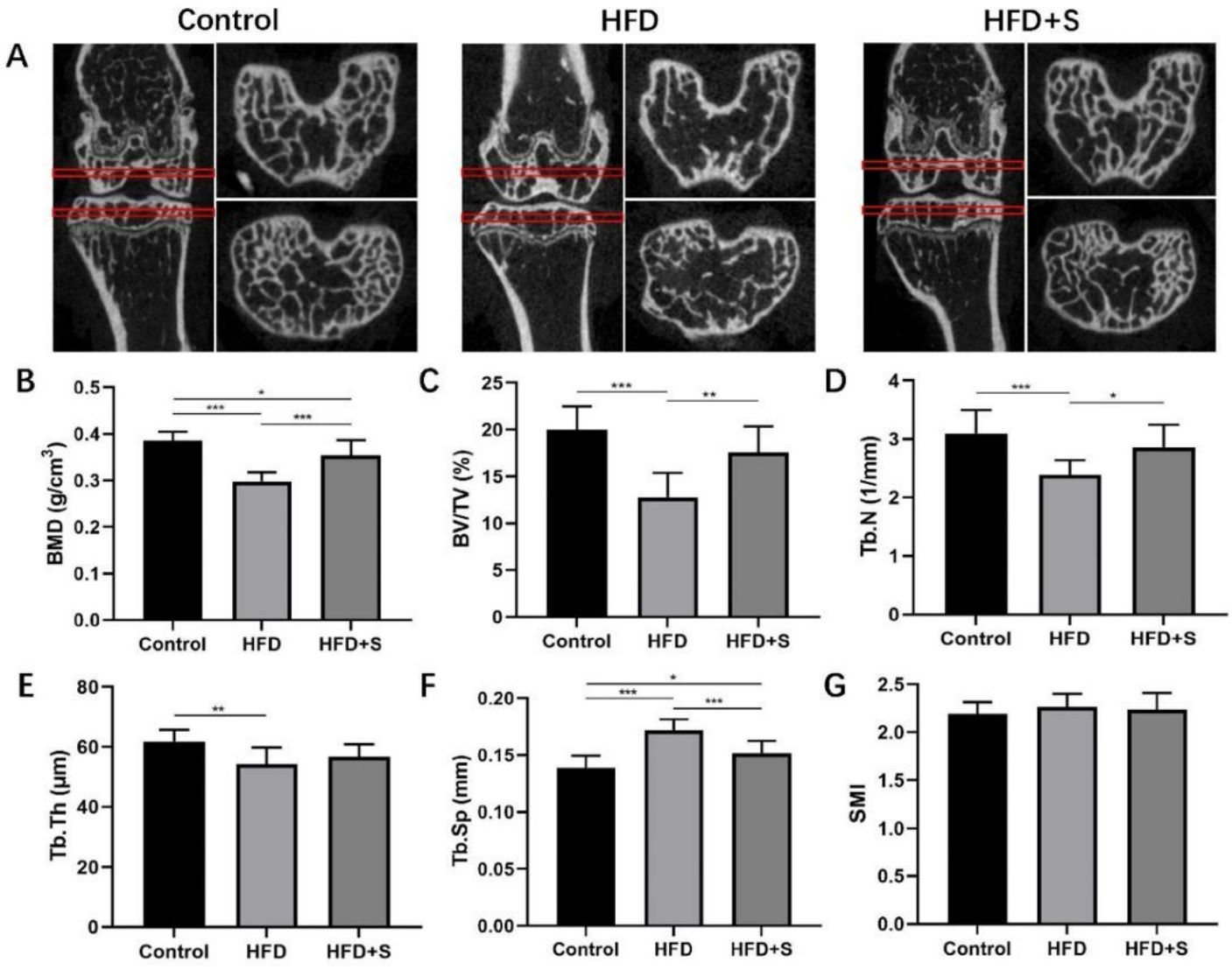

Figure 5

The simvastatin reverses the decreased bone mass and impaired bone microstructures induced by HFD. (A) The representative micro-CT images of the femur and tibial subchondral bone. (B-G) Morphological parameters of the trabecular bone mineral density (BMD), Bone Volume/Total Volume (BV/TV), Trabecular Number (Tb.N), Trabecular Thickness (Tb.Th), Trabecular Separation Distance (Tb.Sp), Structure model index (SMI). Data are expressed as mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001.

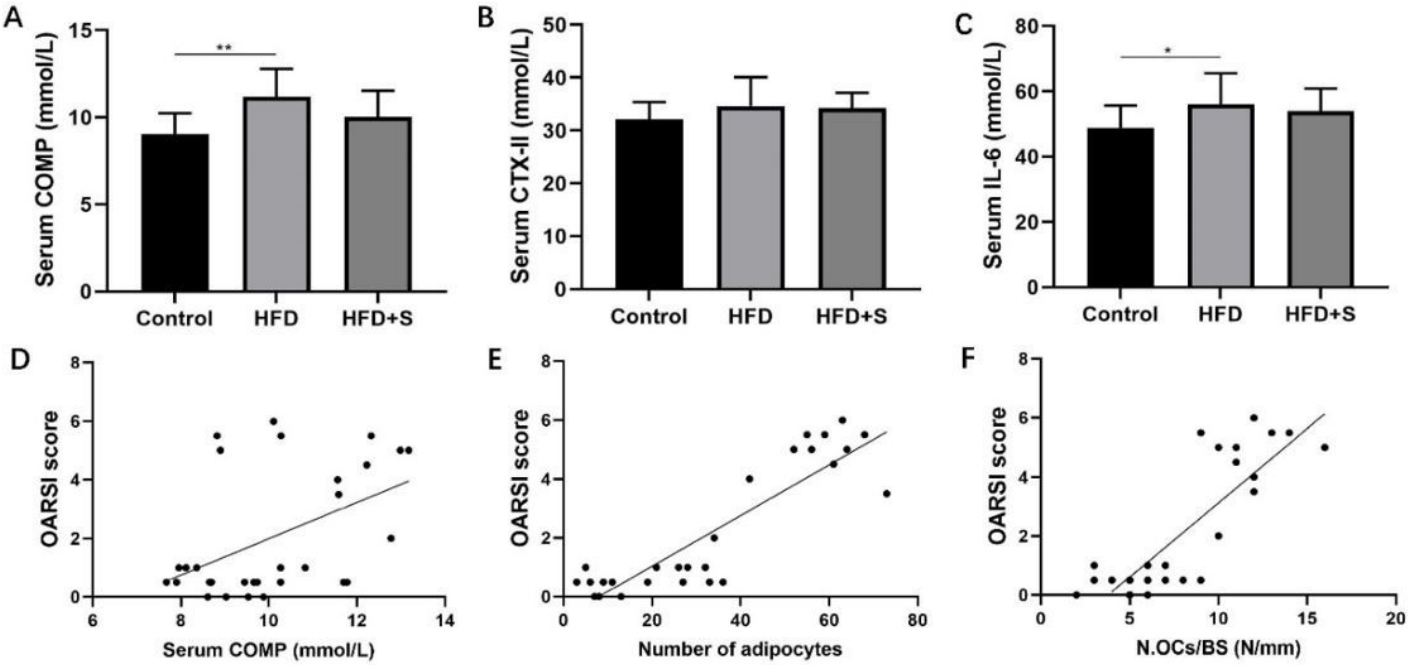

Figure 6

Serum biomarker concentrations and Spearman's rank correlations. (A-C) Serum concentrations of COMP, CTX-II and IL-6 in each group. (D-F) Analysis of correlations between Serum concentrations of COMP, the number of adipocytes in subchondral bone marrow, the number of osteoclasts on trabecular bone surface and OARSI score. The regression line (solid) is shown. Data are expressed as mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMethods.docx
- SupplementaryResult.docx
- SupplementaryTable1.docx