Original Research

Pravastatin prevents steroid-induced osteonecrosis in rats by suppressing PPARγ expression and activating Wnt signaling pathway

Yini Jiang*,[1], Yanqiong Zhang*,[1], Haojun Zhang[2], Bin Zhu[2], Ping Li[2], Chao Lu[3], Ying Xu[1], Weiheng Chen[3] and Na Lin[1]

[1]Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China; [2]Institute of Clinical Medical Sciences, China-Japan Friendship Hospital, No.2 Yinghua Dongjie, Hepingli, Chaoyang District, 100029 Beijing, China; [3]Wangjing Hospital, China Academy of Chinese Medical Sciences, Beijing 100102, China

Corresponding author: Na Lin. Email: linna888@163.com

*These authors as the first authors contributed equally to this article.

Abstract

Steroid-induced osteonecrosis of the femoral head (steroid-induced ONFH) is characterized by increase of intraosseous pressure because of lipid metabolism disturbance such as elevation of adipogenesis and fat cell hypertrophy in the bone marrow, subsequently leading to disturbances of coagulation-fibrinolysis system in the femoral head and finally resulting in bone ischemia. Pravastatin has been demonstrated to be useful in preventing steroid-induced ONFH in animal models. However, its exact mechanisms acting on this disease have not been fully elucidated. To address this problem, steroid-induced ONFH rat model was constructed to evaluate the effects of pravastatin treatment on the osteonecrotic changes and repair processes. Then, Micro-CT-based micro-angiography was performed to assess the effects of pravastatin treatment on vascularization. In addition, serum lipid levels were detected by haematological examination. After that, the expression of peroxisome proliferator-activated receptor gamma (PPARγ), Wnt3a, low density lipoprotein receptor-related protein 5 (LRP5), β-catenin and runt-related transcription factor 2 (RUNX2) at both mRNA and protein levels were further detected by immunohistochemistry, real-time quantitative PCR, and Western blot analyses. The results, the ratio of empty lacuna, adipose tissue area, and adipocyte perimeter in the bone marrow were dramatically lower in the pravastatin treatment groups than in the model group (all $P < 0.05$). Moreover, by micro-CT quantification, pravastatin treatment dose-dependently increased vessel volume, vessel surface, percentage of vessel volume, and vessel thickness of the femoral heads of steroid-induced ONFH rats. Importantly, pravastatin treatment could prevent steroid-induced ONFH by suppressing the expression of PPARγ, and increasing the expression of Wnt3a, LRP5, β-catenin, and RUNX2, at both mRNA and protein levels, in the femoral heads of steroid-induced ONFH rats. In conclusion, Pravastatin may prevent steroid-induced ONFH by suppressing PPARγ expression and activating Wnt signaling pathway.

Keywords: Pravastatin, steroid-induced osteonecrosis of the femoral head, adipogenesis, osteoblastogenesis, peroxisome proliferator-activated receptor gamma, Wnt3a/LRP5/β-catenin/RUNX2 signaling pathway

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Introduction

Steroid-induced osteonecrosis of the femoral head (steroid-induced ONFH) represents a degenerative bone disease characterized by the increase of intraosseous pressure because of lipid metabolism disturbance such as elevating of adipogenesis and fat cell hypertrophy in the bone marrow, subsequently leading to disturbances of coagulation-fibrinolysis system in the femoral head and finally resulting in bone ischemia.[1] The pathogenesis and aetiology of steroid-induced ONFH have not been fully elucidated. The current treatment for this disease simply focuses on preventing irreversible complications, such as biomechanical collapse of the femoral head and osteoarthritis of the hip joint.[2] Thus, it is highly desirable to develop promising pharmacological agents for preventing the onset of steroid-induced ONFH, enhancing bone repair, improving collapse of the articular surface, and keeping away from hip arthroplasty.

Because of the considerable attention on the therapeutic strategies for steroid-induced ONFH, there have been accumulating pharmacological agents for the treatment and

prevention of this disease. They function as lipid-lowing drugs or anticoagulants which address specific physiological risk factors for steroid-induced ONFH, such as lipid emboli, adipocyte hypertrophy, increased intra-osseous pressure, and venous thrombosis.[3] In recent years, statin drugs, as lipid-lowering, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors,[4] have been demonstrated to be effective in the treatment of steroid-induced ONFH. For example, Pengde *et al*.[5] found that lovastatin can prevent development of steroid-induced ONFH in rabbits by inhibiting adipogenesis. Kang *et al*.[6] further demonstrated that the combined treatment with an anti-coagulant (enoxaparin) agent and a lipid-lowering agent (lovastatin) may reduce the incidence of steroid-induced ONFH in rabbits. Nishida *et al*.[7] also indicated that pitavastatin may reduce the risk of steroid-induced ONFH in rabbits. Handal *et al*.[8] indicated that the introduction of atorvastatin calcium in corticosteroid treated rabbits may actually worsen the bone strength loss caused by corticosteroid treatment. Iwakiri *et al*.[9] found that simvastatin and pravastatin may significantly reduce the incidence of steroid-induced ONFH in rabbits. Among these statin drugs, pravastatin is the most extensively studied one in both primary and secondary prevention trials.[10] It is well known because of its efficacy in reducing total cholesterol (TC) and LDL, and rare side effects. Although the therapeutic effects of pravastatin in steroid-induced ONFH have been found recently,[9,11] its exact mechanisms acting on this disease have not been elucidated. In the current study, we examined the preventive effects of pravastatin on the osteonecrotic changes, repair and vascularization processes, and plasma lipid levels in steroid-induced ONFH rats. We also focused on whether pravastatin has any regulatory effects on disbalance of adipogenesis and osteoblastogenesis after steroid treatment.

Materials and methods

The study was approved by the Research Ethics Committee of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China. All animals were treated in accordance with the guidelines and regulations for the use and care of animals of the Center for Laboratory Animal Care, China Academy of Chinese Medical Sciences.

Animals and grouping

Eighty-five 12-week-old male Wistar rats (Cat No. SCXK-(Jun) 2007-004) weighting 300–320 g were obtained from Experimental Animal Centre of Academy of Military Medical Sciences (Beijing, China). All rats were maintained in a room equipped with an air-filtering system, and the cages and water were sterilized.

Groups and treatment

After one week feeding adaptation, the animals were accurately weighed and randomly divided into four groups: control ($n = 20$), model (steroid-induced ONFH rats, $n = 25$), Prava 10 mg/kg (steroid-induced ONFH rats treated with 10 mg/kg pravastatin, $n = 20$), and Prava 20 mg/kg (steroid-induced ONFH rats treated with 20 mg/kg pravastatin, $n = 20$).

Steroid-induced ONFH rat models were constructed according to the previous studies.[12,13] Briefly, methylpednisolone acetate (MPSL; Pfizer Manufacturing BeLgium NV, Puurs, BeLgium) (21 mg/kg) was injected subcutaneously for six weeks to induce osteonecrosis. One hour after the MPSL injection per day, rats in Prava 10 mg/kg and Prava 20 mg/kg groups, respectively, received pravastatin (Daichi-Sankyo Pharmaceutical Co., Ltd., Shanghai, China) dissolved in distilled water by oral gavage at dosages of 10 mg/kg/day and 20 mg/kg/day for six weeks. In the control and model groups, the rats received no treatment. The animals were fed a standard diet and allowed free activity.

Tissue sample preparation

Rats from each group were killed six weeks after the methylpednisolone injection. The rats were anaesthetized with an intravenous injection of Trichloroacetaldehyde hydrate (0.3 mL/kg, sinopharm Chemical Reagent Co., Ltd, China) and were then killed by exsanguination via an aortectomy. For the light microscopic examinations, bilateral femora were obtained at the time of the rat death and the left side were fixed for three days in 4% paraformaldehyde (pH 7.4). The bone samples were decalcified with 10% EDTA for 28 days. Samples were sectioned along the coronal plane for the proximal one-third and cut along the axial plane in the distal part (condyle). Finally, the specimens were embedded in paraffin, cut into 5 μm sections, and stained with haematoxylin and eosin. The right side were stored at −80°C for Western blot and real-time PCR test.

Evaluation of steroid-induced ONFH

The osteonecrotic changes and repair processes in steroid-treated rats were observed by the histopathological examination using a light microscope six weeks after the methylpednisolone injection. The slides were evaluated in a blinded fashion by three independent observers. The evaluation criteria for osteonecrosis were based on the report of Yamamoto *et al*.[14] Osteonecrosis was judged to be present when there was necrosis of medullary hematopoietic cells or fat cells or there were empty lacunae or condensed nuclei in osteocytes. The ratio of empty lacunaes (empty lacunae/the total number of osteocytes) was calculated for each femoral head using a coronal section taken at the maximal femoral width. A computerized image analysis program (image pro 6.0) was used for this calculation.

Quantification and three-dimensional visualization of vessel networks

Femoral head blood vascularization in steroid-treated rats was measured using the microfocal computed tomography (Micro-CT)-based micro-angiography six weeks after the methylpednisolone injection according to the previous studies.[15,16] Briefly, rats from each group were anaesthetized as described above. After perfusion and decalcification, the

femoral shaft was fixed in a polymethylmethacrylate sample tube with its long axis perpendicular to the bottom of the tube in preparation for Micro-CT scanning using μCT (GE Healthcare Biosciences, Piscataway, NJ, USA). The scan was perpendicular to the shaft and was initiated from a reference line 10 mm away from the bottom with an entire scan length of 10 mm. After micro-angiography, the decalcified samples were embedded in paraffin, cut into 5 μm sections, and stained with haematoxylin and eosin for evaluation of steroid-induced ONFH.

Hematological examination

In order to detect the hyperlipidemia-improving effects of pravastatin, the blood samples were collected from the abdominal aorta, six weeks after the methylpednisolone injection. The serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), apolipoprotein A1 (ApoA1), and apolipoprotein B (ApoB) were determined.

Immunohistochemical staining

Paraffin sections (5 μm) of tissue from the femoral head tissues were mounted on poly-L-lysine-coated slides. The paraffin sections were dewaxed by routine method and incubated for 10 min with 3% H_2O_2. The sections were placed in a 37°C, 0.1% trypsinase for 5–30 min for antigen retrieval. Each section was incubated with normal goat serum for 20 min at room temperature, and then with primary antibody against rat PPARγ (rabbit antibody, dilution 1:100, Boster Biotechnology, Inc., Wuhan, China) overnight at 4°C. After incubation with Polymer Helper for 20 min at 37°C, sections were reacted with poly-HRP anti-rabbit IgG for 20 min at 37°C. The sections were then stained with 3, 3-diaminobenzidine (Sigma, St. Louis, MO, USA) and counterstained with hematoxylin.

Specimens were examined using a Leica image analyzer and analyzed by computer image analysis (Leica Microsystem Wetzlar Gmbh., Wetzlar, Germany) in a blinded manner. To localize and identify areas with positively stained cells, 10 digital images per specimen of the femoral head tissues were recorded, and quantitative analysis was performed according to the color cell separation. The results are expressed as the mean region of interest, representing the percentage of area covered with positively stained cells per image at a magnification of 400×.

RNA isolation and real-time PCR

The femoral head tissues were dissected from rats six weeks after the methylpednisolone injection, snap-frozen in liquid nitrogen, ground into powder, and homogenized. The RNA isolation and real-time PCR assay were carried out following the protocol of our previous study.[17] Briefly, total RNA was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) from the tissue homogenates according to the manufacturer's instructions. The total RNA (3 μg) was reverse-transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Thermo Fisher Scientific Inc., CA, USA) according to the instruction manual. The specific transcripts were quantified by quantitative real-time PCR using QuantiTect SYBR Green PCR Kit (Takara bio inc., Tokyo, Japan) and analyzed with ABI 7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA). Gene-specific primers were used for PPARγ (5′-GCG GAA GCC CTT TGG TGA-3′ as forward, and 5′-TGC AGC AGG TTG TCT TGG ATG-3′ as reverse), Wnt3a (5′-GCG TTG GAA CTG CAC CAC TGT-3′ as forward, and 5′-GGC AAA CTC CCG AGA GAC CAT-3′ as reverse), LRP5 (5′-GGC TCG GAT GAA GCT AAC TG-3′ as forward, and 5′-CAG GAT GAT GCC AAT GAC AG-3′ as reverse), β-catenin (5′-CCG AGG ACT CAA TAC CAT TC-3′ as forward, and 5′-CAG ACA TTC GGA ATA GAA CAG-3′ as reverse), RUNX2 (5′-GTA CCC AGG CGT ATT TCA GAT-3′ as forward, and 5′-AGT GAA GGT GGC TGG ATA GTG-3′ as reverse), and GAPDH (5′-ACC CTA AGG CCA ACC GTG AAA AG-3′ as forward, and 5′-CAT GAG GTA GTC TGT CAG GT-3′ as reverse). The mRNA levels of PPARγ, Wnt3a, LRP5, β-catenin, and RUNX2 were normalized to GAPDH mRNA level. PCR was performed as 40 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The relative mRNA expression was calculated with comparative C_T method.

Western blot analysis

The protein expression levels of PPARγ, Wnt3a, LRP5, β-catenin, and RUNX2 in the femoral head tissues obtained from rats in different groups were detected by Western blot analysis. The Western blot protocol and semiquantitative analysis were carried out following the protocol of our previous study.[15] The following antibodies were used: PPARγ antibody (rabbit antibody, dilution 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA,USA), Wnt3a antibody (rabbit antibody, dilution 1:100, Millipore Corporation, Billerica, MA, USA), LRP5 antibody (rabbit antibody, dilution 1:50, Cell Signaling Technology, Inc., Danvers, MA, USA), β-catenin antibody (rabbit antibody, dilution 1:100, Cell Signaling Technology, Inc., Danvers, MA, USA), RUNX2 antibody (rabbit antibody, dilution 1:50, Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH antibody (internal control, rabbit polyclonal antibody, dilution 1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistical analysis

The software of SPSS version13.0 for Windows (SPSS Inc, Chicago, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC, USA) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. For comparisons of means among multiple groups, one-way ANOVA followed by LSD test was performed. Differences were considered statistically significant when $P < 0.05$.

Results

Pravastatin administration reduces osteonecrotic changes in steroid-induced ONFH rats

The osteonecrotic changes and repair processes of rats in each group were histopathologically observed to evaluate the effect of pravastatin administration on steroid-induced ONFH. Compared with the control group, there was an

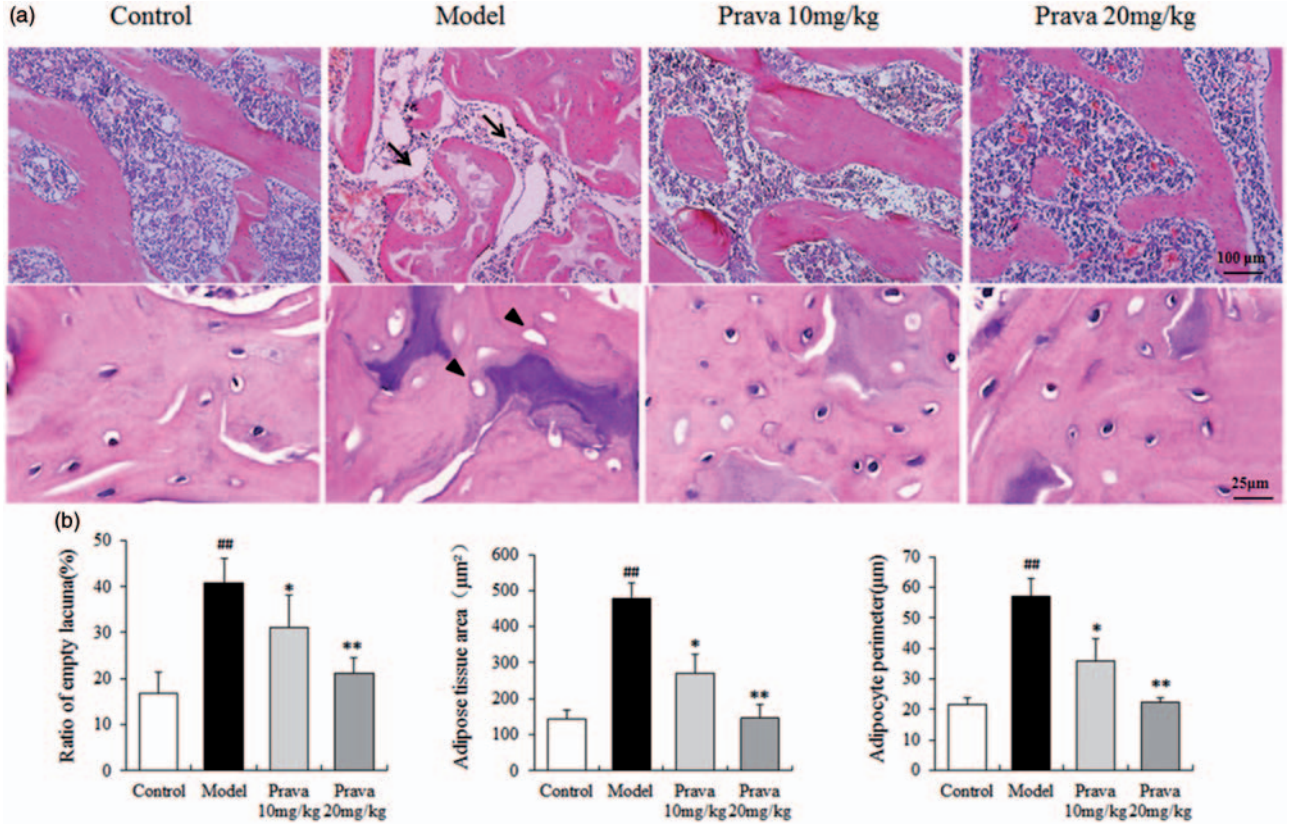

Figure 1 Pravastatin administration enhances osteogenesis and reverses bone marrow adipogenesis. (a) Histological features of the normal bone in normal rat and the osteonecrotic bone in steroid-induced ONFH rats with or without pravastatin administration. (b) Statistical analysis on the differences of the ratio of empty lacuna, the adipose tissue area, and the adipocyte perimeter in the control, model, Prava 10 mg/kg, and Prava 20 mg/kg groups. Data are represented as the mean ± SD ($n = 20$ for control; $n = 25$ for model; $n = 20$ for Prava 10 mg/kg and 20 mg/kg, respectively). $^{\#\#}P < 0.01$ compared with the control group. * and **$P < 0.05$ and $P < 0.01$, respectively, compared with the model group. The arrow heads indicate adipose tissue and the arrows indicate empty lacuna. (A color version of this figure is available in the online journal)

accumulation of bone marrow cell debris found in ONFH lesions in the model group (Figure 1a), while pravastatin administration could dramatically attenuated this change in steroid-induced ONFH rats (Figure 1a). In addition, the ratio of empty lacunaes in the bone trabeculae of the model group was significantly more than that of control group ($40.67 \pm 5.51\%$ vs. $16.67 \pm 4.73\%$, $P < 0.01$, Figure 1(a) and (b)), but was decreased by the treatment of pravastatin with a dose-dependent manner (Prava 10 mg/kg group vs. model group: $31.67 \pm 7.51\%$ vs. $40.67 \pm 5.51\%$, $P < 0.05$; Prava 20 mg/kg group vs. model group: $21.67 \pm 3.61\%$ vs. $40.67 \pm 5.51\%$, $P < 0.01$, Figure 1(b)). Moreover, the adipose tissue area (Prava 10 mg/kg group vs. model group: $269.31 \pm 55.56\ \mu m^2$ vs. $477.67 \pm 44.84\ \mu m^2$, $P < 0.05$; Prava 20 mg/kg group vs. model group: $147.53 \pm 38.04\ \mu m^2$ vs. $477.67 \pm 44.84\ \mu m^2$, $P < 0.01$) and adipocyte perimeter (Prava 10 mg/kg group vs. model group: $36.67 \pm 7.21\ \mu m^2$ vs. $57.23 \pm 6.08\ \mu m^2$, $P < 0.05$; Prava 20 mg/kg group vs. model group: $22.33 \pm 1.52\ \mu m^2$ vs. $57.23 \pm 6.08\ \mu m^2$, $P < 0.01$) in the bone marrow, which were dramatically increased in steroid-induced ONFH rats, were both dose-dependently reduced by the pravastatin treatment (Figure 1b).

Pravastatin administration enhances femoral head neovascularization in steroid-induced ONFH rats

Micro-CT scan was performed to validate the efficiency of pravastatin in steroid-induced ONFH rats. The blood vessel microarchitecture of each group was reconstructed in three dimensions for presentation. As shown in Figure 2(a), the vasculatures in the model group were not visible in and around the necrotic lesion of femoral head, while the samples in the Prava 10 mg/kg group showed lightly increasing capillary vessels and the samples in the Prava 20 mg/kg group showed intensive vascular architecture.

Quantitatively, Figure 2(b) showed that pravastatin treatment dose-dependently increased vessel volume (Prava 10 mg/kg group vs. model group: $0.08 \pm 0.02\ mm^3$ vs. $0.03 \pm 0.05\ mm^3$, $P < 0.01$; Prava 20 mg/kg group vs. model group: $0.10 \pm 0.03\ mm^3$ vs. $0.03 \pm 0.05\ mm^3$, $P < 0.01$), vessel surface (Prava 10 mg/kg group vs. model group: $6.46 \pm 1.45\ mm^2$ vs. $3.51 \pm 0.88\ mm^2$, $P < 0.05$; Prava 20 mg/kg group vs. model group: $9.17 \pm 2.63\ mm^2$ vs. $3.51 \pm 0.88\ mm^2$, $P < 0.05$), percent of vessel volume (Prava 10 mg/kg group vs. model group: $0.05 \pm 0.01\%$ vs. $0.02 \pm 0.01\%$, $P < 0.05$; Prava 20 mg/kg group vs. model group: $0.06 \pm 0.02\%$ vs. $0.02 \pm 0.01\%$, $P < 0.01$), and vessel

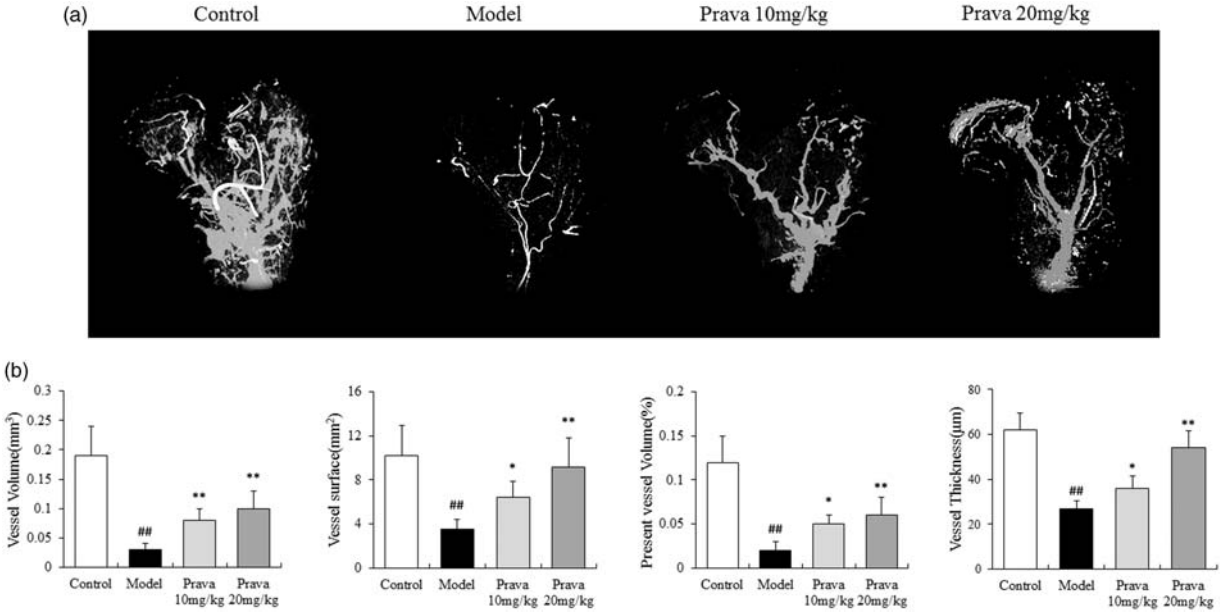

Figure 2 Pravastatin administration enhances femoral head neovascularization. (a) Representative images of micro-CT reconstructed 3-D microangiography of proximal femur from control, model, Prava 10 mg/kg, and Prava 20 mg/kg groups. (b) Statistical analysis on the differences of vessel volume, vessel surface, percentage of vessel volume, and vessel thickness in the femoral heads of steroid-induced ONFH rats in different groups. Data are represented as the mean ± SD ($n = 10$). $^{\#\#}P < 0.05$ compared with the control group. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared with the model group

thickness (Prava 10 mg/kg group vs. model group: 36.12 ± 5.36 μm vs. 27.4 4 ± 7.42 μm, $P < 0.05$; Prava 20 mg/kg group vs. model group: 54.81 ± 7.63 μm vs. 27.44 ± 7.42 μm, $P < 0.01$) of the femoral heads of steroid-induced ONFH rats.

Pravastatin administration improves hyperlipidemia in steroid-induced ONFH rats

In the lipid system, the blood chemistry data showed that steroid hormone administration (model group) induced marked hyperlipidemia, such as significantly elevated TC (compared with control group: 1.81 ± 0.09 mmol/L vs. 1.34 ± 0.23 mmol/L, $P < 0.01$, Figure 3(a)), TG (compared with control group: 0.52 ± 0.09 mmol/L vs. 0.20 ± 0.09 mmol/L, $P < 0.01$, Figure 3(b)), LDL (compared with control group: 0.70 ± 0.18 mmol/L vs. 0.7351 ± 0.12 mmol/L, $P < 0.01$, Figure 3(c)), ApoA1 (compared with control group: 1.69 ± 0.19 g/L vs. 1.34 ± 0.20 g/L, $P < 0.01$, Figure 3(e)), and ApoB (compared with control group: 1.84 ± 0.34 g/L vs. 1.30 ± 0.28 g/L, $P < 0.01$, Figure 3(f)) levels, but significantly decreased HDL levels (compared with control group: 0.30 ± 0.06 mmol/L vs. 0.58 ± 0.14 mmol/L, $P < 0.05$, Figure 3(d)). More interestingly, doses of 10–20 mg/kg pravastatin could dose-dependently improve hyperlipidemia by decreasing TC (all $P < 0.01$, Figure 3(a)), TG (all $P < 0.01$, Figure 3(b)), LDL (all $P < 0.05$, Figure 3(c)), ApoA1 (all $P < 0.05$, Figure 3(e)) and ApoB (all $P < 0.05$, Figure 3(f)) levels, and increasing HDL levels (all $P < 0.05$, Figure 3(d)).

Pravastatin administration suppresses PPARγ expression in the femoral heads of steroid-induced ONFH rats

The expression patterns and subcellular localization of PPARγ protein in the femoral heads of steroid-induced ONFH rats were examined by immunohistochemistry analysis. As shown in Figure 4(a), PPARγ protein was detected in the femoral head samples of steroid-induced ONFH rats with strong immunoreactivity and located in nuclei. Higher expression of PPARγ was observed in the model group than that in control group (mean ± S.D.: 60.11 ± 12.24% vs. 24.05 ± 5.62%, $P < 0.01$, Figure 4(a)). With the treatment of pravastatin (doses of 10–20 mg/kg), the increased immunoreactivity of PPARγ protein was significantly reduced (Prava 10 mg/kg group vs. model group: 41.23 ± 12.22% vs. 60.11 ± 12.24%, $P < 0.05$; Prava 20 mg/kg group vs. model group: 25.27 ± 7.55% vs. 60.11 ± 12.24%, $P < 0.01$; Figure 4(a)). More interestingly, compared with the control group, PPARγ mRNA level in the femoral heads of steroid-induced ONFH rats in the model group was dramatically increased (mean ± S.D.: 1.05 ± 0.18 vs. 0.25 ± 0.13, $P < 0.01$, Figure 4(b)), while doses of 10–20 mg/kg pravastatin significantly reduced the expression of PPARγ mRNA (Prava 10 mg/kg group vs. model group: 0.84 ± 0.19 vs. 1.05 ± 0.18, $P < 0.05$; Prava 20 mg/kg group vs. model group: 0.63 ± 0.12 vs. 1.05 ± 0.18, $P < 0.01$; Figure 4(b)), which was consistent with the changes into PPARγ protein expression levels in different group as shown in Figure 4(c).

Pravastatin administration activates Wnt3a/LRP5/β-catenin/RUNX2 signaling pathway in the femoral heads of steroid-induced ONFH rats

We further detected the changes into Wnt3a, LRP5, β-catenin, and RUNX2 expression at both mRNA and protein levels in the femoral heads of steroid-induced ONFH rats with or without pravastatin administration in order to evaluate its influence in this pathway. As shown in Figure 5(a), pravastatin treatments markedly increased the expression of Wnt3a, LRP5, β-catenin, and RUNX2 at mRNA

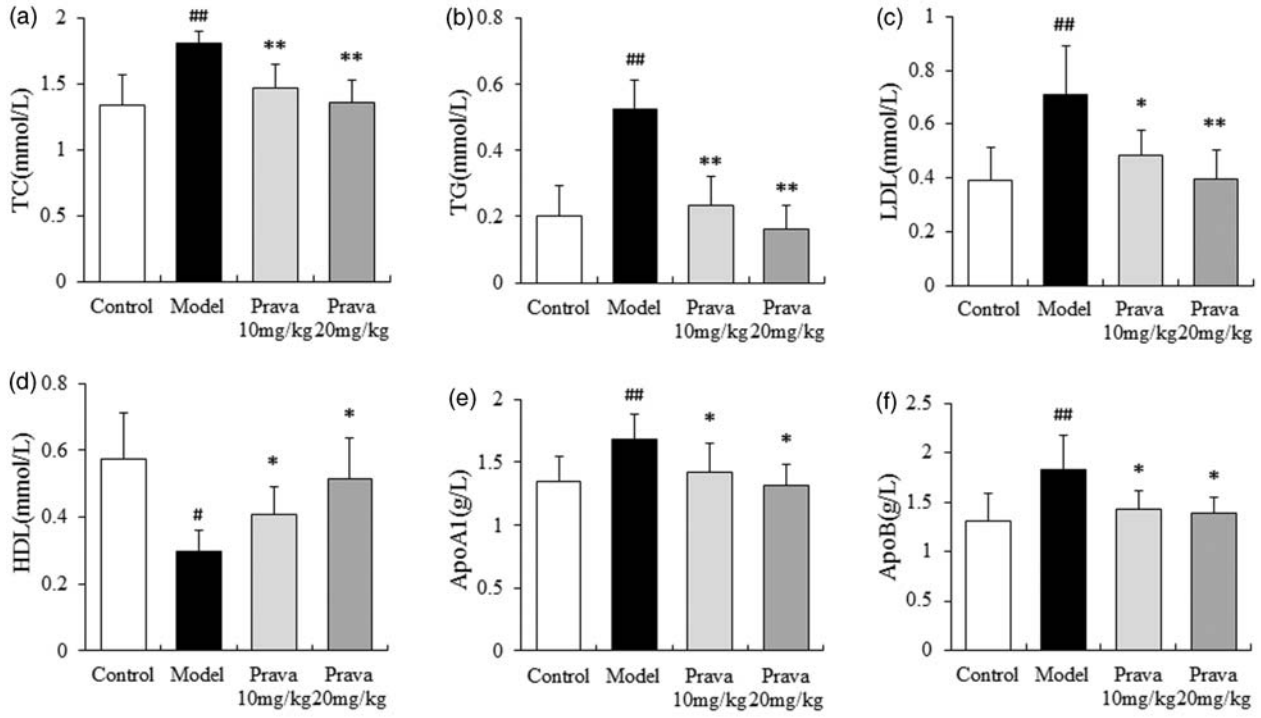

Figure 3 Pravastatin administration improves hyperlipidemia in steroid-induced ONFH rats. The steroid hormone administration (model group) induced marked hyperlipidemia, such as significantly elevated TC (a), TG (b), LDL (c), ApoA1 (e), and ApoB (f) levels, but significantly decreased HDL levels (d). More interestingly, doses of 10–20 mg/kg pravastatin could significantly reduce hyperlipidemia by decreasing TC (a), TG (b), LDL (c), ApoA1 (e), and ApoB (f) levels, and increasing HDL levels (d). Data are represented as the mean ± SD ($n = 10$ for control; $n = 15$ for model; $n = 10$ for Prava 10 mg/kg and 20 mg/kg, respectively). $^{\#}$ and $^{\#\#}$ $P < 0.05$ and $P < 0.01$, respectively, compared with the control group. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared with the model group

levels, which was reduced in the femoral heads of steroid-induced ONFH rats (all $P < 0.05$, Figure 5(a)). In line with the findings of quantitative real-time RT-PCR, Wnt3a, LRP5, β-catenin, and RUNX2 protein expression levels detected by Western blot analysis were also activated by doses of 10–20 mg/kg pravastatin treatments significantly with a dose-dependent tendency (all $P < 0.05$, Figure 5(b)).

Discussion

Steroid treatment is one of the most comment risk factors associated with osteonecrosis.[18] Prolonged steroid use produces a hyperlipidemic state in most patients and subsequently results in abnormal coagulopathy and bone marrow fat-cell packing, leading to microvascular occlusion and high intra-osseous pressure, all of which put them at risk for osteonecrosis.[19] Therefore, accumulating researchers postulate that a lipid-lowering agent may prevent the conditions associated with the development of osteonecrosis. As lipid-clearing agents, statin agents have been demonstrated to reverse the adverse effects of steroid treatment on lipid metabolism, thus reducing the severity and frequency of ONFH.[4–11] In the current study, the main findings are as follows: (1) pravastatin attenuates steroid-induced ONFH by reducing the osteonecrotic changes and the bone marrow adipogenesis of steroid-induced ONFH rats; (2) pravastatin enhances femoral head neovascularization and improves hyperlipidemic state of steroid-induced ONFH rats; and (3) pravastatin suppresses the expression of PPARγ and activates Wnt/LRP5/β-catenin/RUNX2 signaling pathway in the femoral heads of steroid-induced ONFH rats.

Pravastatin is abundantly prescribed for the treatment of dyslipidemia and to reduce the risk of cardiovascular disease.[10] Because of its efficiency to enhance the antioxidant activity and local lipid kinetics by directly acting on adipocytes and blood vessels, pravastatin has been used to attenuate the development of ONFH.[9, 11] In steroid-induced ONFH rats, we found that the ONFH lesions present histological characteristics of empty lacunae accompanied by surrounding marrow cell necrosis and occupation of adipocytes, which are the main features at early stage of ONFH. After the treatment of pravastatin, the ratio of empty lacunaes and the area of bone marrow occupied by adipocytes were significantly reduced, suggesting the improvement in local lipid metabolism by pravastatin administration. Since the impeded blood flow through the femoral head is incriminated in the aetiopathogenesis of steroid-induced ONFH, our study here applied a novel technique, using Micro-CT-based micro-angiography, to visualize and quantify new blood vessel formation and vascularization in rat femoral head. Recent studies have demonstrated that this technique is quantitative and effective for assessing vascularization.[15,16] Our data demonstrated a significant increase in blood vessel volume, vessel surface, percentage of vessel volume, and vessel thickness in the pravastatin treatment groups, suggesting pravastatin may dose-dependently increase vascularization of the femoral heads in rat model.

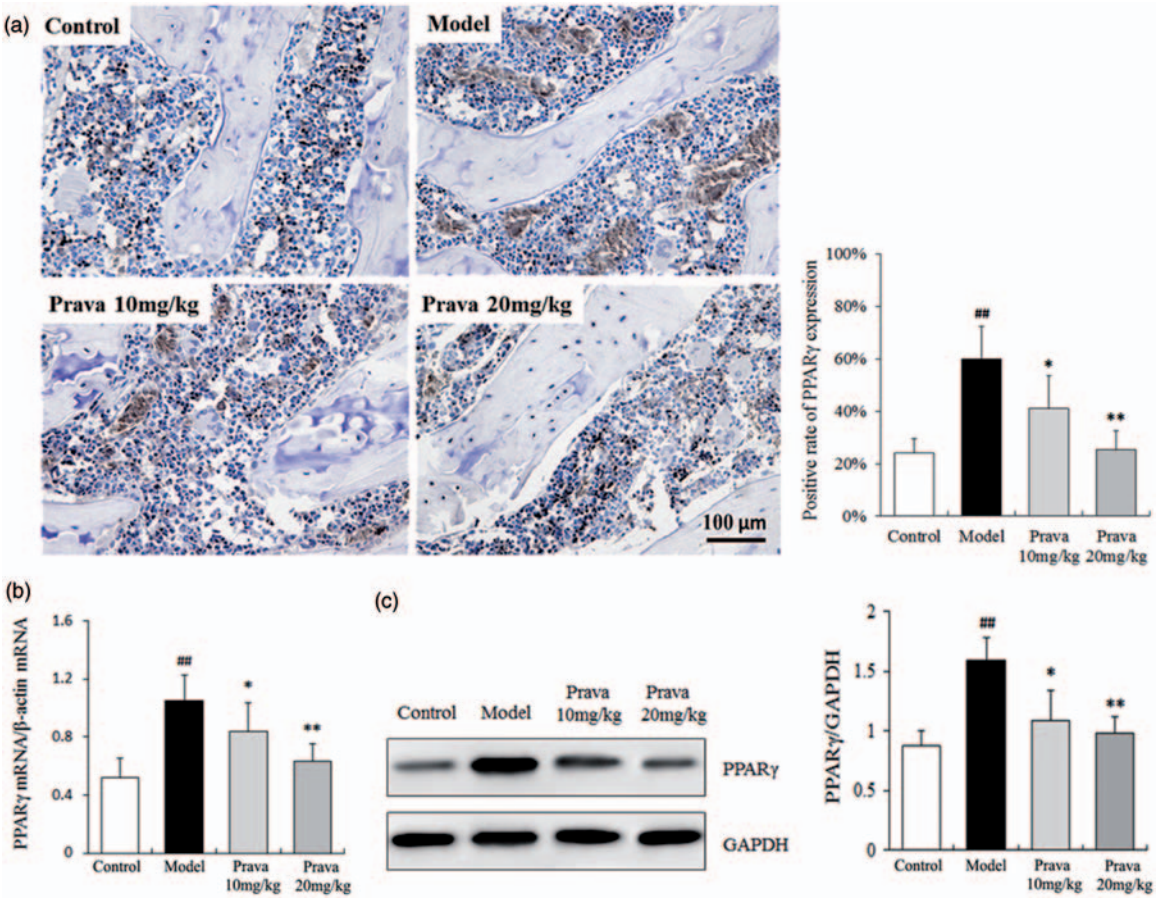

Figure 4 Pravastatin administration suppresses PPARγ expression in the femoral heads of steroid-induced ONFH rats. The mRNA and protein expression levels of PPARγ in the femoral heads of steroid-induced ONFH rats in control, model, Prava 10 mg/kg, and Prava 20 mg/kg groups were, respectively, detected by immunohistochemistry (a), quantitative real-time RT-PCR (b), and Western blot (c). Data are represented as the mean ± SD ($n = 20$ for control; $n = 25$ for model; $n = 20$ for Prava 10 mg/kg, and 20 mg/kg, respectively). $^{\#\#}P < 0.01$, compared with the control group. * and **', $P < 0.05$ and $P < 0.01$, respectively, compared with the model group. (A color version of this figure is available in the online journal.)

To the best of our knowledge, this is the first time to reveal the effect of pravastatin on vascularization in steroid-induced ONFH models. These findings are in line with previous data showing the effect of pravastatin on the improvement of islet vascularization[20] and on the enhancement of myocardial vascularization with decrease in interstitial fibrosis and attenuating cardiomyocyte and glomerular loss.[21] Pravastatin may improve vascular function independently from their lipid-lowering effects by activating the PI3K/Akt pathways,[20] which needs further validation in steroid-induced ONFH model. Moreover, it is not surprising to find that pravastatin as a lipid-lowering agent can decrease the serum lipid levels in steroid-induced ONFH rats. Functionally, pravastatin inhibits the action of HMG-CoA reductase or inhibits the synthesis of very low density lipoproteins, which are the precursor to LDL. These reductions increase the number of cellular LDL receptors and, thus, LDL uptake increases, removing it from the blood stream, subsequently, leading to the reduction in circulating TC, LDL, and TG, and an increase in HDL.[22]

The dysregulation of homeostasis of bone is one of the main pathogenesis in steroid-induced ONFH. Steroids may induce adipogenesis, decrease osteogenesis in bone marrow stroma cells, and produce intracellular lipid deposits resulting in death of osteocytes.[23] Adipogenic differentiation is a complex process regulated by many factors. PPARγ, as a member of the nuclear receptor gene superfamily of ligand-activated transcription factors, functions as a stimulator of bone marrow adipogenesis and an inhibitor of osteoblastogenesis. Previous study found that PPARγ activation *in vivo* may promote osteoclast-mediated bone resorption in a receptor-dependent manner.[24] Given the close association between adipocyte and osteoblast formation, it is reasonable to speculate that the potential exists to prevent or treat bone loss by inhibiting bone marrow adipogenesis. Our study found that the increased expression of PPARγ at both mRNA and protein levels occurred in the steroid-induced ONFH rat models, suggesting steroid treatment may induce the bone marrow cells to change from a primarily osteogenic nature to adipogenic phenotype. With the pravastatin administration, the expression of PPARγ was significantly decreased. Both the canonical and noncanonical Wnt pathways, which have been reported to positively regulate osteoblast differentiation and inhibit adipocyte differentiation, are also essential mediators in the development of steroid-induced ONFH.[25] Georgiou

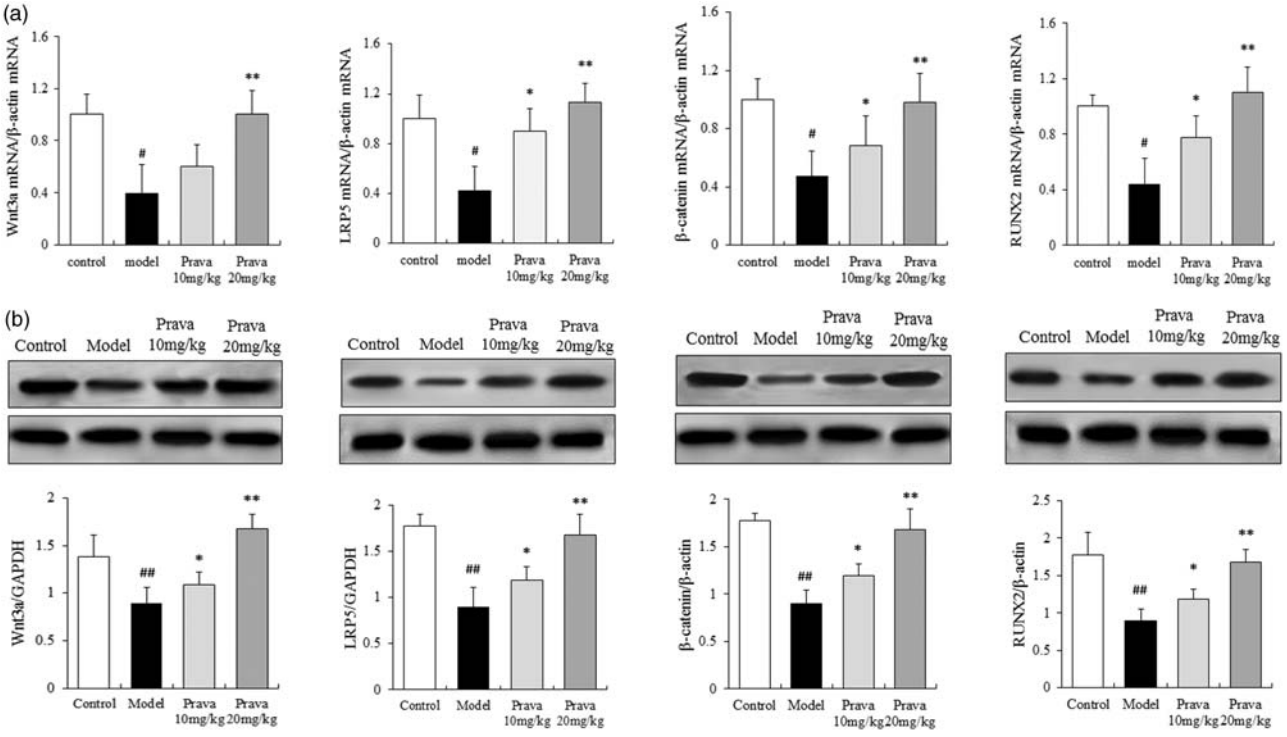

Figure 5 Pravastatin administration activates Wnt3a/LRP5/β-catenin/RUNX2 signaling pathway in steroid-induced ONFH rats. Wnt3a, LRP5, β-catenin, and RUNX2 expression at both mRNA and protein levels in the femoral heads of steroid-induced ONFH rats with or without pravastatin administration were detected by quantitative real-time RT-PCR (a), Western blot (b). Data are represented as the mean ± SD ($n = 20$ for control; $n = 25$ for model; $n = 20$ for Prava 10 mg/kg and 20 mg/kg, respectively). $^{\#\#}P < 0.01$ compared with the control group. * and **, $P < 0.05$ and $P < 0.01$, respectively, compared with the model group

et al. also found that cancer drug Methotrexate treatment may cause bone loss and bone marrow adiposity,[26] and Wnt/β-catenin signaling defect may mediate methotrexate-induced bone/fat changes.[27] The Wnts (Wnt-1, Wnt-3 a, Wnt-5 b, Wnt-7 a and Wnt-10 b) bind to membrane-bound frizzled receptors and LRP5/6 co-receptor initiating a signaling cascade that leads to nuclear accumulation of β-catenin. Binding of Wnt ligands to frizzled receptors and LRP co-receptors inhibits cytoplasmic degradation of β-catenin and stimulates its translocation into the nucleus. Nuclear β-catenin binds to and co-activates members of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors. The β-catenin–TCF/LEF complexes bind to specific recognition elements in the promoters of target genes (such as RUNX2) and modulate their expression.[25] Steroids treatment has been demonstrated to affect the Wnt signaling pathway and reduce bone formation by inhibiting the activity of β-catenin and regulating the expression of Wnt signal-related molecules in osteoblasts,[25] in line with which, we in the current study found the decreased expression of members in Wnt pathways, including Wnt3a, LRP5, β-catenin, and RUNX2. Moreover, our data show that pravastatin administration could activate the expression of these members at both mRNA and protein levels. Recent studies have demonstrated that the Wnt signaling can induce osteoblastogenesis through transrepression of PPARγ via the histone methyltransferase complex.[28] In this respect, we speculate that pravastatin might activate the Wnt pathway which switches the cell fate decision from adipocytes to osteoblasts by suppressing the transactivation function of PPARγ.

In conclusion, our data offer the convincing evidence for the first time that pravastatin may prevent steroid-induced ONFH by suppressing PPARγ expression and activating Wnt signaling pathway. These findings highlight the regulatory effects of pravastatin on the disbalance of adipogenesis and osteoblastogenesis during the progression of steroid-induced ONFH.

AUTHOR CONTRIBUTIONS

NL, YJ, and YZ participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; YJ, HZ, BZ, PL, CL, and YX conducted the experiments; WC participated in the analysis of the data; and YZ and YJ wrote the manuscript.

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