

RESEARCH ARTICLE

Methylprednisolone Inhibits Autophagy of Vascular Endothelial Cells in Rat Femoral Head *Via* PI3K/Akt/mTOR Pathway

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Objective: To study the relationship between vascular endothelial cells (VEC) and autophagy, and its regulatory mechanism in steroid-induced avascular necrosis of the femoral head (SANFH).

Methods: In cell experiment, VEC were isolated and cultured from the femoral head of Sprague–Dawley rats and divided into three groups: blank control group (Ctrl), methylprednisolone group (MP), and methylprednisolone+mTOR-shRNA group (MP + shmTOR). The autophagy formation was observed by transmission electron microscope. The mRNA expression of PI3K, Akt, mTOR, Beclin1 and MAP1LC3 was detected by RT-PCR and the protein expression was detected by Western blot and immunofluorescence. Expression of the damage marker 6-keto-PGF1 α was detected by the ELISA method. *In vivo* experiment, after establishing the model, the grouping method was the same as cell experiment. Autophagosomes were observed by same method, and the expression of related factors was detected by the same method in cell experiment.

Results: In the cell experiment, autophagosomes in the MP group were significantly lower than in the Ctrl group, and the autophagosomes in the MP + shmTOR group were intermediate between two groups ($P < 0.05$). The mRNA expression levels of PI3K, Akt and mTOR in the MP group were significantly higher than in the Ctrl group, while the MP+ shmTOR group presented intermediate levels between these groups (average gray value were 3837.90, 2996.30, 3005.60, $F = 428.64$, $P < 0.05$). mRNA expression levels of Beclin1 and MAP1LC3 in the MP group were significantly lower than that in Ctrl group ($P < 0.05$). The content of 6-keto-PGF1 α in the MP + shmTOR group was higher than in the Ctrl group and lower than in the MP group at the evaluated time intervals (average absorbance value were 104.98, 206.83, 145.91, $F = 352.83$, $P < 0.01$). *In vivo* experiment, the content of 6-Keto-PGF1 α in the hormone group increased as time went on; the mTOR-si group was higher than that in control group, but lower than that in the hormone group ($P < 0.01$). The mRNA expressions of Beclin1 and MAP1LC3 in the control group were higher than those in the hormone group, while the mRNA expressions of PI3K, Akt and mTOR were lower than those in the mTOR-si group ($P < 0.05$).

Conclusion: The steroid inhibited the physiological protective effect of autophagy on SANFH by increasing the expression of PI3K/Akt/mTOR signaling pathway related factors and decreasing the expression of Beclin1 and MAP1LC3 in the femoral head VEC.

Key words: Autophagy; PI3K/Akt/mTOR; RNA interference; Steroid-induced avascular necrosis of the femoral head; Vascular endothelial cells

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Introduction

Steroid-induced avascular necrosis of the femoral head (SANFH) has been recognized since it was first reported in 1957.¹ SANFH has a complex biological process, its pathogenesis is unknown and there is no effective treatment available. Previous studies have shown that programmed cell death (autophagy and apoptosis) of osteoblasts is associated with SANFH. Steroid-induced damage to the blood supply of the femoral head is the main cause of SANFH.² Vascular endothelial cell (VEC) injury is the most important pathological change observed in SANFH, but its exact regulatory mechanism needs to be further clarified. The blood supply of the femoral head has particularities and comes mainly from the obturator, medial and lateral circumflex femoral arteries. However, the supply range of these arteries is limited, the collateral circulation is small and the corresponding supply area is prone to blood supply disturbance after injury.³ VEC originates from the mesoderm angioblasts and is lined on the medial side of the vascular lumen. VEC has many functions, such as vascular regulation, coagulation, fibrinolysis, immunity, substance transport and release of bioactive substances, which are closely related to hemodynamics.^{4,5}

The main factors that cause VEC injury include mechanical, immune and chemical factors, oxidative stress, steroid levels, tobacco and toxins; however, VEC death mode and its regulation are still unclear.⁶ Autophagy is an important pathway for the metabolism of intracellular material and an important part of the degradation/recycling system that exists widely in single-celled organisms, plants and mammalian cells. The imbalance of autophagy regulation mechanism is related to aging and the occurrence and development of many diseases, such as tumor, inflammatory and autoimmune diseases.⁷ Cardiovascular disease has been found to be associated with VEC dysfunction caused by oxidative stress, which is characterized by excessive peroxide production and organelle damage. Physiological autophagy can remove damaged organelles and increase cellular resistance to stress. Therefore, decreased autophagy can lead to oxidative stress and blood vessel aging.⁸ Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian rapamycin target protein (mTOR) is an important signaling pathway for regulating autophagy (PI3K/Akt/mTOR). mTOR can regulate cell growth and proliferation, and is also a key regulator in the early stage of autophagy. The expression levels of autophagy-related genes or proteins Beclin 1, LC3-II, Atg5-Atg12 and typical autophagosomes in macrophages increased significantly, when mTOR was silenced by mTOR-siRNA.⁹

The hypothesis of this study is to: (i) propose that steroid affects the expression of factors related to the regulation of PI3K/Akt/mTOR autophagy; (ii) VEC damage is caused by inhibiting the protective effect of physiological autophagy on blood vessels or by increasing pathological autophagy; and (iii) mTOR-shRNA can reverse some consequences of VEC dysfunction such as abnormal hemodynamics and the decrease of blood flow, which leads to ischemia, hypoxia and necrosis of the femoral head.

Materials and Methods

Cell Experiment

Vascular Endothelial Cells (VEC) Culture

The femoral head of 8-week-old Sprague-Dawley (SD) rats (Qinglongshan Animal Breeding Farm, Nanjing, China) was removed and the cancellous bone in the femoral head was bitten into bone particles by a rongeur. Subsequently, these bone particles were placed in the serum-free DMEM medium (Hyclone, Logan, UT, USA) and digested with 5 ml of 2% type I collagenase (Nanjing Shengxing Co. Ltd., Nanjing, China) for 1 hour. After digestion, the culture medium was removed and 0.1% trypsin (Hyclone, Logan, UT, USA) was added to digest again for 5 min. The digested cell solution was filtered twice in a 70 μ m cell sieve (Bio-rad, Hercules, CA, USA). Subsequently, this solution was centrifuged at 2000 rpm for 10 min and the cell precipitates were collected. After the cells had grown to 90%, the culture medium was removed and the cells were washed twice with PBS (Nanjing Shengxing Co. Ltd., Nanjing, China). After removing PBS, 1 ml of trypsin was added for 2 min of digestion and then 3 ml of complete culture medium (Nanjing Shengxing Co. Ltd., Nanjing, China) was added to neutralize trypsin and stop digestion. The digested cells were transferred to a 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 min. After the supernatant was discarded, the suspension cells in 3 ml medium were added and subcultured in a petri dish at 1:2. VEC was detected by immunofluorescence in the later stage.

Cytoviral Infection

After the cell digestion with trypsin, the cells were counted by blood cell counting board (Nanjing Shengxing Co. Ltd., Nanjing, China). 5×10^5 cells were inoculated on a plate, completely blown away, fully mixed and cultured in an incubator (Thermo Fisher, Waltham, MA, USA). After overnight culture, the complete medium was removed and the medium without antibiotics (Sciencell) and adenovirus (Shanghai Jima Pharmaceutical, Shanghai, China) according to MOI titer 1:100 were added. The cells were gently shaken, placed in an incubator and after 6 h, the virus-containing medium was removed. After being cultured in complete medium for 24 h, the follow-up test could be performed. The cultured VEC were divided into control group (Ctrl), methylprednisolone group (MP), and methylprednisolone combined with mTOR-shRNA adenovirus transfection group (MP + shmTOR, NC:AGCTGTGCCCTTCATCCCT, shmTOR:CCCAGCCTTTGTCATGCCT). In the MP group, only methylprednisolone was added (final concentration in the culture medium of 1 μ mol/L). In the MP + shmTOR group, methylprednisolone and shRNA adenovirus were added (final concentration of 1 μ mol/L and 0.5 mol/L, respectively).

Immunofluorescence

Paraformaldehyde 4% (Nanjing Shengxing Co. Ltd., Nanjing, China) was used to fix the cells for 10 min at room temperature. After removing paraformaldehyde, cells were washed three times with deionized water and cell membrane was

punctured with PBS containing 0.5% Triton X100 (Alladin Co. Ltd, Shanghai, China) on the ice for 10 min. Then, PBS-Triton was removed and the cells were washed three times with PBS. PBS-3% BSA (blocking solution) was added for 30 min in dark at room temperature. Antibodies to CD31 (Abcam, Shanghai, China), Factor VIII (Novus, Shanghai, China), PI3K (Sigma-Aldrich, St. Louis, MO, USA), Akt (Sigma-Aldrich, St. Louis, MO, USA), mTOR (Sigma-Aldrich, St. Louis, MO, USA), LC3 (Sigma-Aldrich, St. Louis, MO, USA) and Beclin1 (Sigma-Aldrich, St. Louis, MO, USA) were diluted in PBS-1% BSA solution (Sigma-Aldrich, St. Louis, MO, USA) in a 1:100 ratio. The blocking solution was removed and the primary antibody (Invitrogen, Carlsbad, CA, USA) was added. After incubation at 4°C overnight, the primary antibody was removed and the cells were washed three times with PBS-0.35% Tween-20 (PBS-T). The secondary fluorescent antibody (Invitrogen, Carlsbad, CA, USA) was diluted in PBS-1% BSA in a 1:400 ratio, added after PBS-T removal and incubated for 1 h at room temperature. Then, the secondary antibody was removed and the cells were washed three times with PBS-T. After removing PBS-T, a 100 ng/mL DAPI solution (Invitrogen, Carlsbad, CA, USA) was added and incubated for 10 min at room temperature. Then, DAPI was removed and the cells were washed again three times with PBS-T. Finally, the fluorescence microscope (Nikon) was used to visualize the labeled cells after adding anti-fluorescence quenching solution (Invitrogen, Carlsbad, CA, USA).

ELISA

VEC were cultured for 12, 24, 48 and 72 h. The supernatant was collected, placed in an EP tube and centrifuged at 12,000 rpm for 10min at 4°C. The Biotin-labeled antibody, HRP-labeled antibiotic protein and standard protein were diluted according to the kit instructions (Cusabio, Wuhan, China) and the reaction was performed in accordance with the operation instructions. At the end of the reaction, the absorbance of each pore was detected sequentially at the wavelength of 450 nm after 5 min of incubation with the enzyme labeling instrument (Nanjing Shengxing Co. Ltd., Nanjing, China).

Electron Microscope

VEC was removed from the culture medium and 2.5% glutaraldehyde was added to fix the cells at 4°C overnight. Subsequently, cells were washed with PBS for 10 min and fixed with 1% osmium tetroxide (Nanjing Shengxing Co. Ltd., Nanjing, China) for 1 h at room temperature. Then, the cells were covered with 10% gelatin and fixed with glutaraldehyde (Nanjing Shengxing Co. Ltd., Nanjing, China) at 4°C for 1 h. Then, the samples were dehydrated with an increasing concentration of ethanol solution (30%, 50%, 70%, 90%, 95%, 100%, 100%, 100%). After soaking and embedding with epoxy resin, the samples were sliced with Leica UC6 ultra-thin slicer (Leica, Wetzlar, Germany). Finally, under the condition of 110kV, the samples were observed and photographed by transmission electron microscope (JEOL Co. Ltd., Beijing, China).

RT-PCR

VEC was removed from the culture medium and washed with PBS. After centrifugation, PBS was removed and cells were incubated with 1 ml of Trizol (Takara, Dalian, Japan) for 2 min at room temperature. Then, 200 µL of chloroform, were added and after 15 s of agitation, samples were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected, an equal volume isopropanol was added and the samples were mixed by turning the tube upside down at room temperature for 5 min. Then, the samples were centrifuged at 4°C for 10 min and supernatant was discarded. 1 ml of 75% ethanol (Shanghai Aladdin Biochemistry, Shanghai, China) was added to the precipitated, and after slightly shaking for 15 s, samples were centrifuged again at 4°C for 5 min. After removing supernatant, on a super clean table, the precipitation blast in the tube was dried for 5 min and then dissolved in 50 µl of DEPC water and stored at -80°C. According to the reaction system, various related reagents are added to the eight connecting tubes. The preparation of the reagents for the entire system needs to be operated on ice. The reaction system is mixed gently, the eight connecting tubes are covered with a lid and the adherent liquid is precipitated at the bottom by instantaneous centrifugation. The eight tubes were placed in the PCR instrument (ABI, Veriti, Waltham, MA) for detection and discarded at the end of the reaction. The data were analyzed directly according to the values collected and displayed by the PCR instrument.

Western Blot

After VEC was cultured to the specified time point, the medium was removed and washed with PBS. The medium was removed and 100 µL RIPA lysate was added to the ice lysate for 30 min. After lysate was completed, the lysate was transferred to a 1.5 ml centrifuge tube and centrifuged at 12,000 rpm at 4°C for 5 min. After centrifugation, the supernatant was transferred to a 1.5 ml centrifuge tube and stored in a refrigerator at -20°C. Then determine the protein content, take 10 µL standard protein diluted to 100 µL with PBS, make the final concentration 0.5 mg/mL. Add standard to standard well of 96-well plate by 0, 1, 2, 4, 8, 12, 16, 20 µl, then add solution to 20 µl, dilute standard. Add appropriate volume of sample to each sample well of 96-well plate, and likewise add solution to 20 µL. Add 200 µl BCA solution to each well, place at 37°C for 30 min, and calculate the protein concentration according to the standard curve (unit: mg/ml).

After the preparation of the samples, SDS-PAGE electrophoresis and membrane transfer were carried out. Finally, immune reaction was performed. First, the PVDF membrane was moved to the sealing solution and shaken at room temperature for 1 h. Then the primary antibody was incubated, diluted with TBST solution containing 5% BSA at 1:1000, and incubated overnight at 4°C. Then washed with TBST shaker three times, 10 min each. Finally, the second antibody was incubated, diluted with TBST at 1:5000 and incubated for 2 h at room temperature. After that, the second antibody

was washed with TBST for three times at room temperature, 10 min each, and chemiluminescence reaction could be performed. The experimental results were photographed by Tanon gel imager and the images were obtained.

Animal Experiment

Modeling

Sixty healthy and clean SD rats (Qinglongshan Animal Breeding Farm, Nanjing, China) were selected, with no restriction on gender, standard diet, body weight 250 ± 20 g, and one in each cage. Randomly divided into three groups. The first group was the control group, A total of 20 rats were intramuscularly injected with normal saline, 2 ml/ time, three times in

total, 24 h interval; Twenty rats in the MP group were intramuscularized with methylprednisolone (albicin biotechnology co., Ltd., Shanghai, China) for three times at an interval of 24 h. Twenty rats in MP + mTOR-si group were intramuscular injected with methylprednisolone (20 mg/kg) and simultaneously injected with cationic liposome (gima pharmaceutical co., Ltd., Shanghai, China) into mTOR-siRNA (0.5 mol/kg) for three times at an interval of 24 h. The experimental animals in each group were sacrificed at 1, 2, 3 and 4 weeks, and five animals were sacrificed at each time.

Specimen Preparation

After each rat was sacrificed, the main blood vessels of the femoral head and both hind limbs were removed under

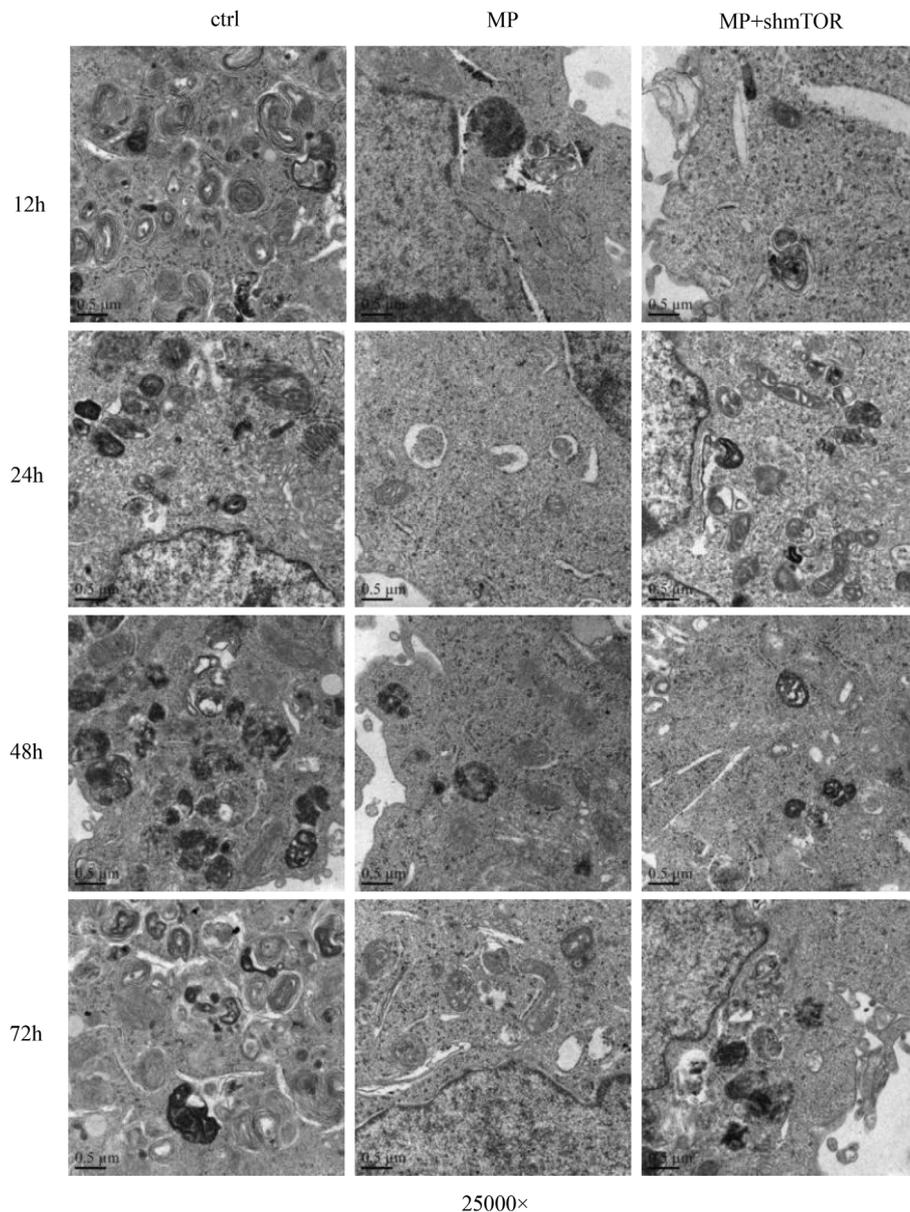


Fig. 1 The formation of autophagosome in VEC was observed by transmission electron microscope. Autophagosome number in the MP + shmTOR group was intermediate between the Ctrl and MP groups

aseptic conditions. MSCT (Philips China, Shanghai, China) tested these changes before each death. Femoral head specimen: one part was fixed with 40% neutral formaldehyde, dehydrated, and when 15%HYLINE> the other part was fixed with 5% glutaraldehyde (Shanghai Chemical Reagent Co., Ltd., Shanghai, China) solution. Bone blocks of about 1 mm × 1 mm × 1 mm were cut at 0.2–0.3 cm below the cartilage of the femoral head, and decalcated with 5% nitric acid for 2–3 h. Then it was fixed with 1% osmium tetroxide (Shanghai Chemical Reagents Co., Ltd., Shanghai, China) for 1 hour and embedded with EPON812 (epoxy resin). The embedding agent and anhydrous ethanol (Sinophem Chemical Reagents Co., Ltd., Shanghai, China) were mixed at the concentrations of 1:1, 2:1 and 3:1, respectively. Anhydrous ethanol was replaced layer by layer. Ultrathin sections with a thickness of 50 nm were prepared by soaking for 24 h in a 37°C incubator and polymerizing for 48 h in a 60°C oven. The ultrathin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (JEM011) and scanning electron microscopy for immunofluorescence electron microscopy. The blood vessel samples were divided into three parts: the first part was fixed with 40% neutral formaldehyde, dehydrated, 15%EDTA decalcified, gradient alcohol dehydrated, embedded in wax

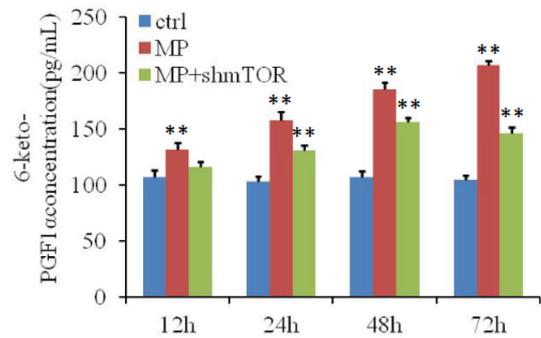


Fig. 3 Detection of 6-keto-PGF1 α expression by ELISA of three experimental groups (Ctrl MP and MP + shmtOR) ** represents $P < 0.01$

immersion, stained and made into 5 μ m thick sections for light microscopy observation. The second part was fixed with 5% glutaraldehyde solution, 1% osmium tetroxide solution (Shanghai Chemical Reagent Co., Ltd., Shanghai, China) for 1 h, gradient ethanol dehydration, embedding EPON812 (epoxy resin), embedding agent and anhydrous ethanol in 1:1, 2:1 and

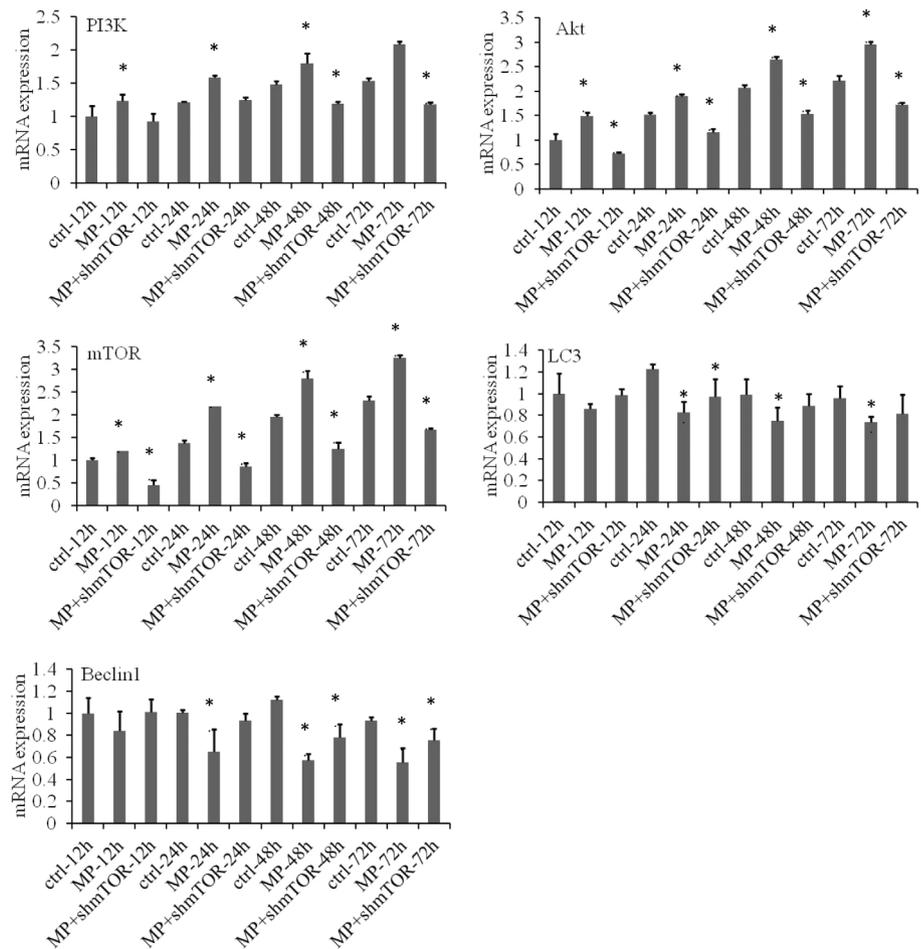


Fig. 2 mRNA expression changes of PI3K, Akt, mTOR, Beclin1 and LC3 were quantitatively detected by RT-PCR. ** represents $P < 0.01$ and * represents $P < 0.05$

3:1 concentrations, layer by layer to replace anhydrous ethanol, embedding, Ultrathin slices with thickness of 50 nm were prepared by soaking for 24 h in 37 incubator and polymerizing for 48 h in 60 oven. Transmission electron microscopy, scanning electron microscopy and immunofluorescence electron microscopy (uranyl acetate and lead citrate double staining) were performed. In the third part, VEC was extracted and the mRNA and protein expressions of Beclin1, MAP1LC3, IFT20, OFD1Beclin1, MAP1LC3, PI3KI, mTOR, Akt and autophagic cell death were detected.

Statistical Analysis

SPSS22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis in cell and animal experiment. LSD test was used to compare the data between the two groups and one-way ANOVA was used to compare the data between multiple groups.

The vacancy rate of bone lacunae was compared by Chi-square test. P values <0.05 were considered statistically significant.

Results

Cell Experiment Result

mTOR-shRNA Increases VEC Autophagy

We identified by immunofluorescence, the expression of VEC markers CD31 and Factor VIII in cultured cells, which attests that they are VEC. VEC autophagy was observed by transmission electron microscope. As shown in Fig. 1, autophagosomes can be seen in the visual field of the three experimental groups. Physiological autophagy intensity was found in the Ctrl group and the number of autophagosomes in the MP group was significantly lower than in the Ctrl group. The autophagosome

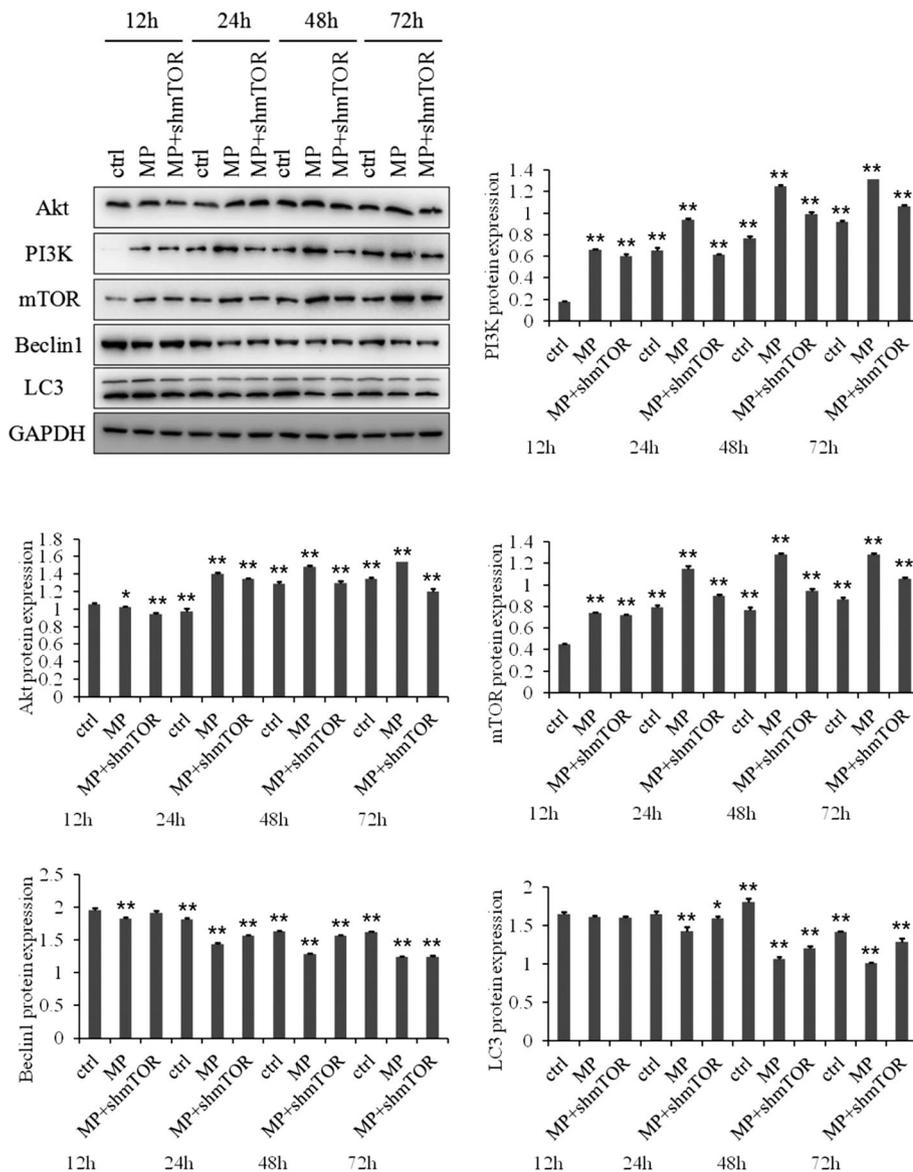


Fig. 4 Protein expression changes of PI3K, Akt, mTOR, Beclin1 and LC3 detected by Western Blot (* $P < 0.05$; ** $P < 0.01$ vs control, $n=3$)

number in the MP + shmTOR group was intermediate between the two groups, indicating that methylprednisolone can inhibit VEC autophagy and that the longer its action time, the more obvious the effect of inhibiting autophagy. MTOR-shRNA could down-regulate mTOR expression, decrease the inhibitory effect of methylprednisolone on autophagy and increase the level of autophagy. Even so, autophagy in the MP + shmTOR group was lower than in the Ctrl group.

Methylprednisolone Increases PI3K/Akt/mTOR Related Factors Expression

To determine the effect of methylprednisolone on mRNA expression of PI3K/Akt/mTOR-related factors and autophagy-related genes in VEC, RT-PCR were used to determine the mRNA expression levels of PI3K, Akt, mTOR, Beclin1 and MAP1LC3 in VEC, Western Blot and immunofluorescence were used to detect protein expression. As shown in Fig. 2, the mRNA expression levels of PI3K, Akt and mTOR in MP group was significantly higher than in the Ctrl group, while the MP + shmTOR group presented intermediate levels between these groups. MRNA expression levels of Beclin1 and MAP1LC3 in the MP group was significantly lower than that in the Ctrl group. Once again, the MP + shmTOR group presented intermediate levels. Analysis of VEC culture after 12, 24, 48 and 72 h showed that PI3K/Akt/mTOR-related factors gradually increased and autophagy-related genes decreased in

the MP group, while PI3K/Akt/mTOR-related factors decreased and autophagy-related genes gradually increased in the MP + shmTOR group.

mTOR-shRNA Can Repair VEC Injury

VEC damage was evaluated by ELISA and using 6-keto-PGF1 α as damage marker. The results showed that the 6-keto-PGF1 α content in the Ctrl group was stable and did not change after 12, 24, 48 and 72 h of VEC culture, while the 6-keto-PGF1 α content in the MP group gradually increased over time. The content of 6-keto-PGF1 α in the MP + shmTOR group was higher than in the Ctrl group and lower than in the MP group at the evaluated time intervals. It is noteworthy that, after 72 h of culture, the expression in the MP group was twice that of the control group, and the 6-keto-PGF1 α content in the MP group was almost twice as high as in the Ctrl group (Fig. 3). These results, combined with those in Figs 2–4, suggest that methylprednisolone reduces the physiological autophagy of VEC, which consequently suffer greater damage without the protection provided by it. On the other hand, mTOR can partially repair VEC injury and physiological autophagy. Our data showed that mTOR-shRNA can regulate mTOR *via* the PI3K/Akt/mTOR pathway, decreasing mRNA expression of PI3K, Akt and mTOR and increasing mRNA expression of Beclin1 and MAP1LC3.

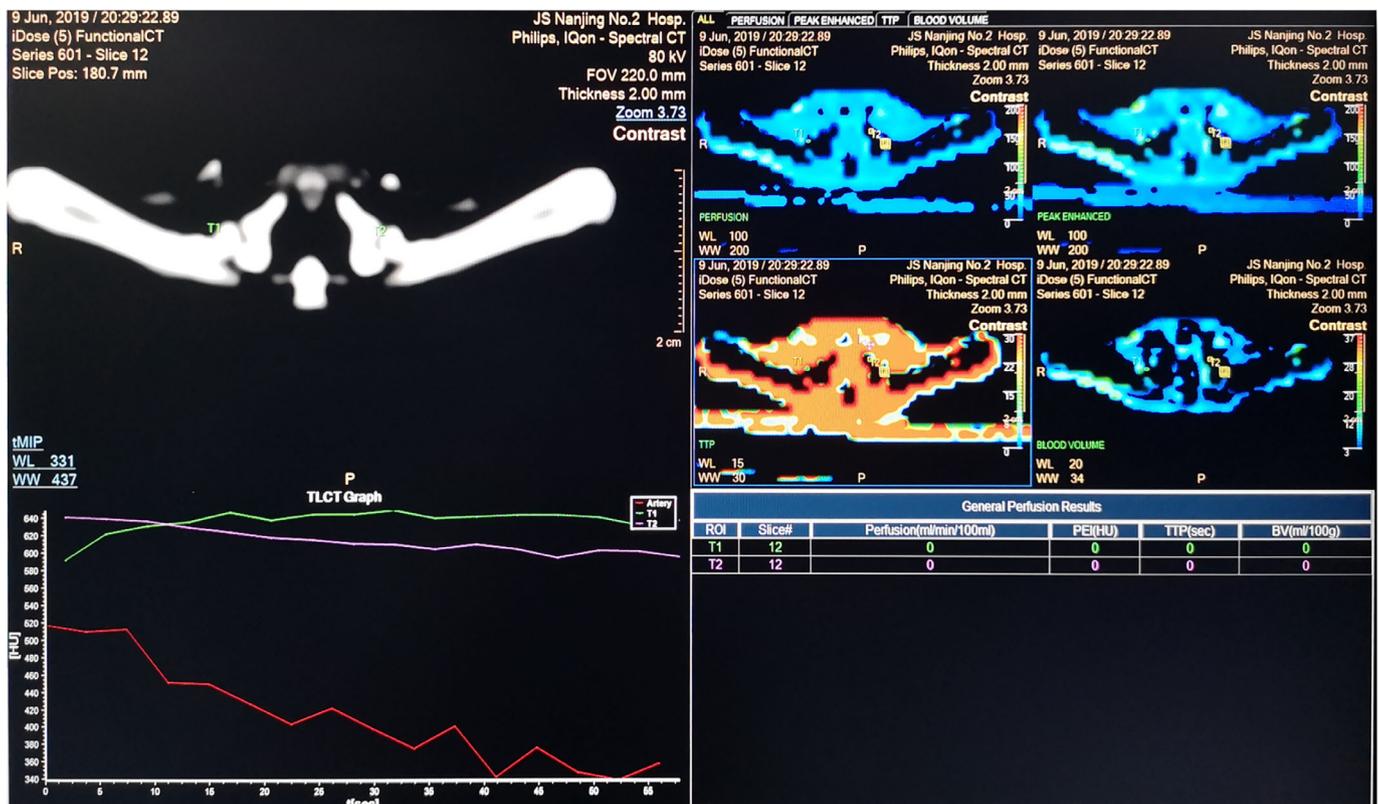


Fig. 5 Multi slice CT detection result of the rat femoral artery

Animal Experiment Result

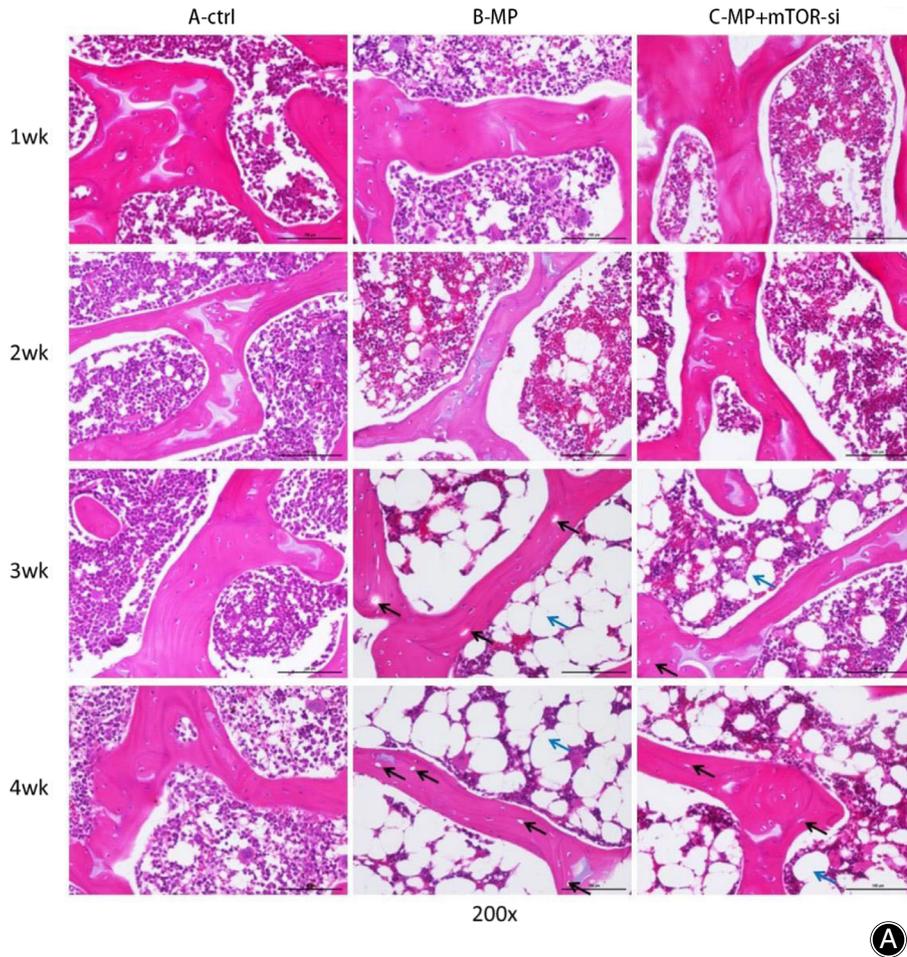
MSCT Examination

On the basis of SANFH animal model induced by hormones, MTOR-SiRNA was introduced into the cationic liposome as a carrier, and blood perfusion parameters such as rBF, rBV and MTT were measured by MSCT to determine the local hemodynamic changes of femoral head (Fig. 5). However, due to the

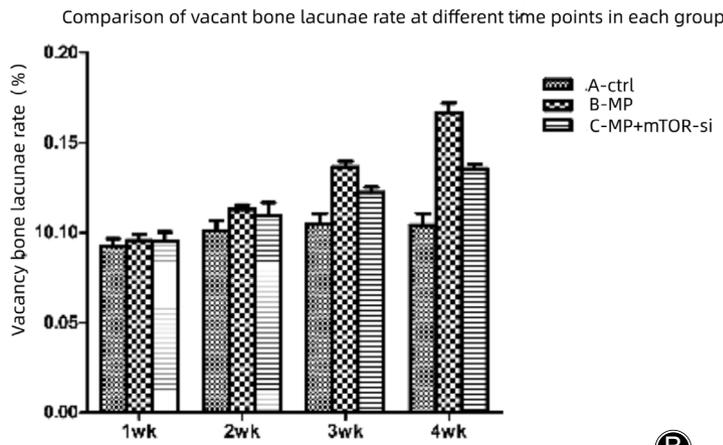
small diameter of the bone supplying artery in rats, the blood flow changes in the femoral artery were not detected successfully.

mTOR -siRNA Mitigates Hormone Damage to Bone Cells

The ischemic necrosis of bone cells and bone tissue in each group was observed by light microscope. As can be seen from Fig. 6, no significant difference was observed between each group in the first 2 weeks. In the control group,



(A)



(B)

Fig. 6 (A) Comparison of HE staining morphology of the femoral head in each group. (B) The ratio of vacant lacunae at different time points

hematopoietic cells were abundant in the medullary cavity, bone trabecular structure was clear and orderly, fat cells were few, and hollow bone lacunae were rare. After 3 weeks in b-methylprednisolone group, the number of adipocytes in the bone marrow lumen increased significantly, accompanied by hyperplasia, some osteocytes steatosis, and more vacant bone lacunae. After 3 weeks, the number of fat cells and hollow bone lacunae in c-methylprednisolone + MTOR-SI group was less than that in methylprednisolone group, but more obvious than that in the control group. It showed that bone cells were damaged by methylprednisolone, and the longer the treatment time, the more obvious the damage. The expression of mTOR was inhibited by MTOR-SiRNA, which reduced the destruction effect of hormone on bone cells. However, bone destruction was still observed due to the continued presence of hormone compared with the control group.

mTOR-siRNA Can Partially Repair VEC Damage by Enhancing the Autophagy Pathway of VEC

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to detect the formation of VEC autophagosomes and determine whether there was autophagic cell death.

The electron microscopy showed that 4 weeks after modeling, autophagosomes were visible in the field of vision in the control group, with autophagy phenomenon and clear and complete vascular endothelial cell structure (Fig. 7). In the methylprednisolone treatment group, the nuclei of vascular endothelial cells were dissolved, the mitochondria in cytoplasm were swollen, and the mitochondrial crest was broken until it disappeared completely. No autophagosomes were observed in the visual field. In the methyl prednisolone + MTOR-SI group, mitochondrial swelling was also observed in vascular endothelial cells, but the structure was still discernible, and no autophagosome appeared under visual field. Again, VEC autophagy can be inhibited by methylprednisolone, and the inhibitory effect of autophagy becomes more significant with the extension of action time. However, the expression of mTOR was inhibited by MTOR-SiRNA, which reduced the inhibitory effect of hormone on autophagy and enhanced autophagy, but its level was still lower than that of physiological autophagy.

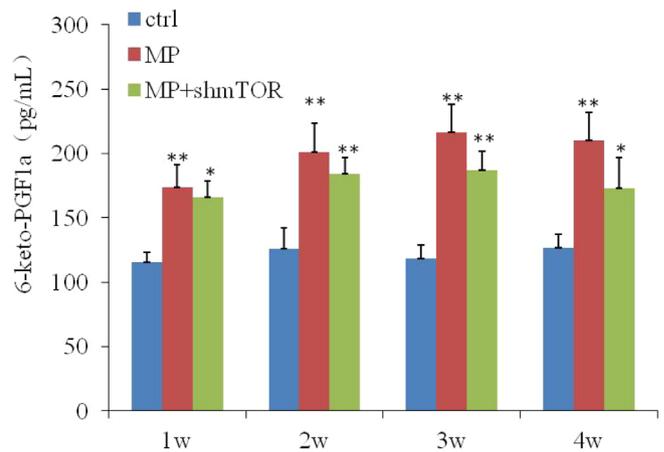


Fig. 8 Expression changes of 6-Keto-PGF1 α detected by ELISA (* $P < 0.05$, ** $P < 0.01$ vs control, $n = 3$)

We used ELISA to detect 6-Keto-PGF1 α (a marker of VEC injury) to determine whether VEC was damaged by hormones and the effect of MTOR-SiRNA on it. As shown in Fig. 8, the content of 6-Keto-PGF1 α in the control group did not change over time, while that in the hormone group showed an overall trend of increase over time, indicating that hormones could damage VEC and the damage would worsen over time. The 6-Keto-PGF1 α content in the siRNA group was higher than that in the control group and lower than that in the hormone group, indicating that VEC damage in the siRNA group was also impaired, but less so than that in the hormone group, indicating that VEC damage caused by hormones could be partially reversed by MTOR-SiRNA. However, due to the continued presence of hormones, VEC damage could not be completely repaired. It suggested that MTOR-SiRNA could partially repair the damage of VEC and enhance the level of VEC autophagy, but the level was still lower than that of physiological autophagy.

Methylisolone Inhibits the Expression of Beclin1 and MAP1LC3 Related Factors

Immunofluorescence staining (GFP) was used to detect GFP-LC3, GFP-Beclin1, GFP-PI3KI, GFP-AKTR and GFP-MTOR

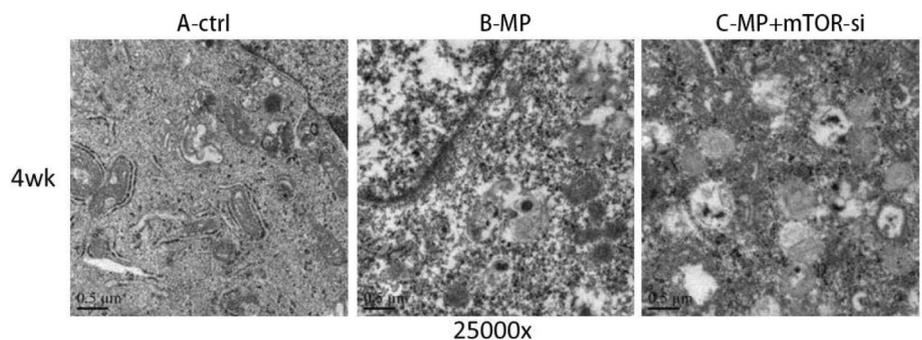


Fig. 7 Formation of autophagosomes under transmission electron microscopy

fusion proteins. The mRNA expressions of MAP1LC3, Beclin1, PI3KI, Akt and mTOR were detected by RT-PCR. The protein expressions of MAP1LC3, Beclin1, PI3KI, Akt and mTOR were quantitatively detected by Western blotting. As can be seen from Figs 9 and 10, the mRNA and protein expressions of Beclin1 and MAP1LC3 in the control group were higher than those in the hormone group, while the mRNA and protein expressions of PI3K, Akt and mTOR were lower, indicating a higher level of VEC autophagy in the control group and the hormone group, and indicating that hormone can inhibit VEC autophagy. MRNA and protein expressions were detected at the time points of 12, 24, 48 and

72 h, respectively. The expressions of Beclin1 and MAP1LC3 in the hormone group showed a decreasing trend, while the expressions of PI3K, Akt and mTOR showed an increasing trend, indicating that the physiological autophagy level of VEC decreased with the passage of hormone action. It is once again proved that hormone can inhibit VEC autophagy.

As shown in Figs 9 and 10, the mRNA and protein expressions of mTOR, PI3K and Akt in the hormone group were higher than those in the MTOR-SiRNA group, while the mRNA and protein expressions of MAP1LC3 and Beclin1 were lower than those in the MTOR-SiRNA group, indicating that the autophagy level of the hormone group was lower than

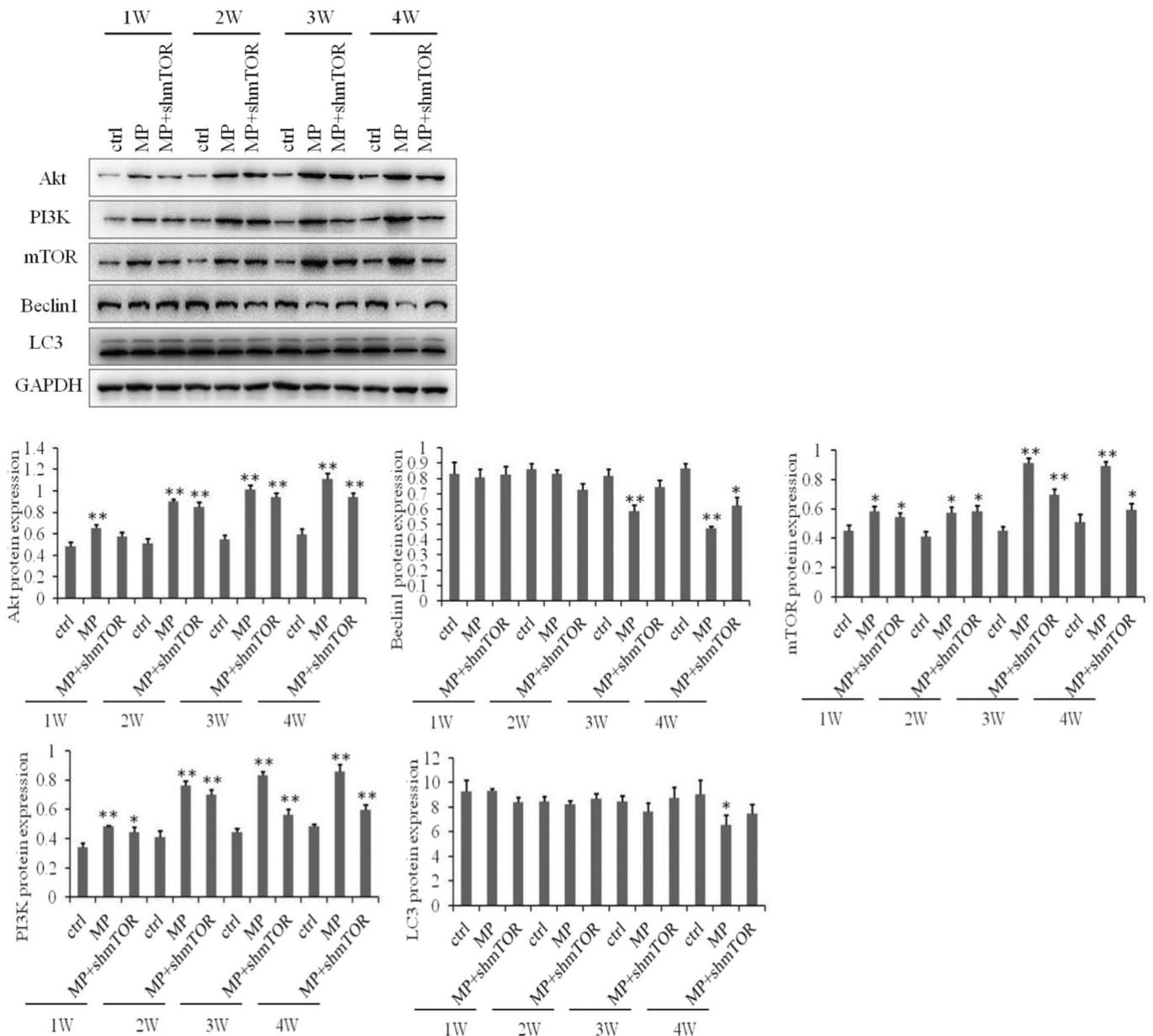


Fig. 9 Expressions of Beclin1, MAP1LC3, Akt, mTOR and PI3KI proteins detected by Western Blot (* $P < 0.05$, ** $P < 0.01$ vs control, $n = 3$)

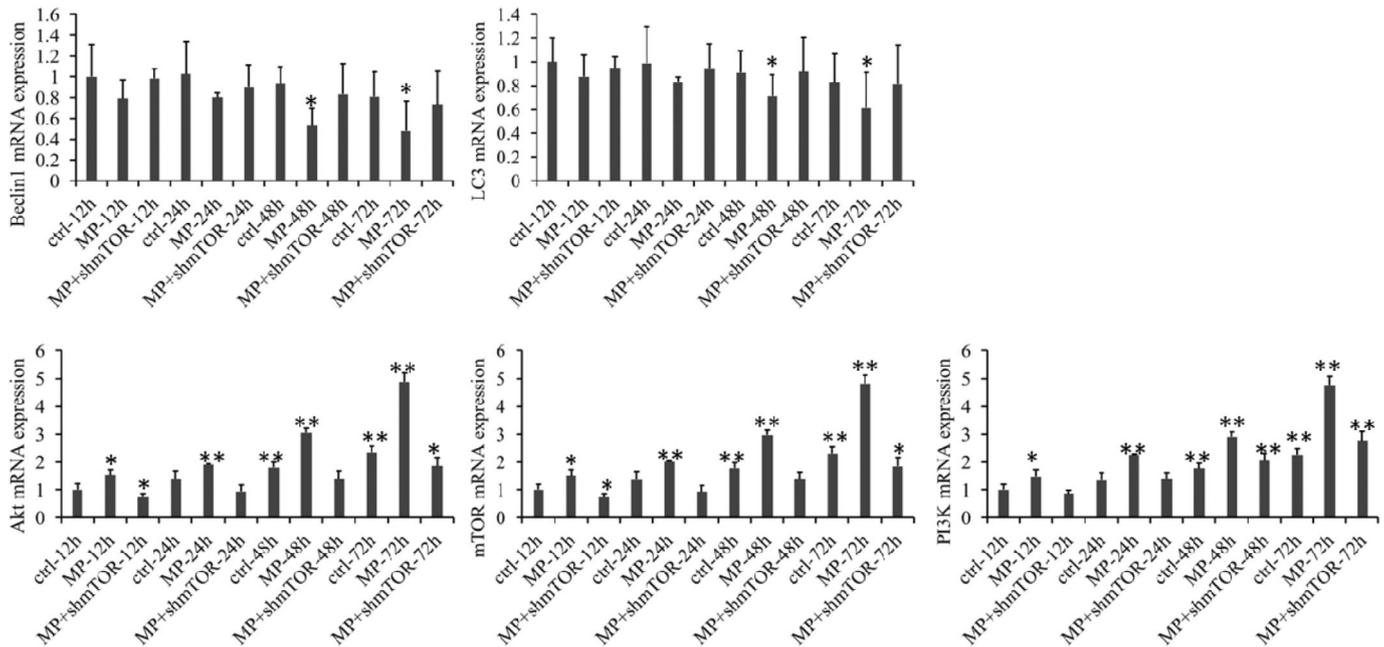


Fig. 10 Expression of Beclin1, MAP1LC3, Akt, mTOR and PI3K genes detected by RT-PCR (* $P < 0.05$, ** $P < 0.01$ vs control, $n = 3$)

that of the MTOR-SiRNA group. MTOR -siRNA can increase the autophagy of VEC. Meanwhile, we also observed that the expressions of PI3K, Akt, mTOR and 6-Keto-PGF1 α in mTOR-SiRNA group increased with time, while the expressions of Beclin1 and MAP1LC3 decreased with time.

Discussion

Vascular Lesion Is the Main Cause of SANFH

There are an estimated 8.12 million cases of osteonecrosis of the femoral head (ONFH) in China; where ONFH incidence rates in different population subgroups are 5.53–11.76/100 000.¹⁰ The increase in incidence may be due to the increased use of adjuvant therapy (e.g., corticosteroids). Unfortunately, osteonecrosis is generally progressive, requires multiple surgeries and usually leads to arthroplasty.¹¹ Although many theories have been proposed, no pathophysiological mechanism has been confirmed as the cause of ONFH. However, it is already known that its basic mechanism involves disorders of blood circulation in specific areas, which eventually leads to necrosis. The blood supply of the femoral head has its particularities since the corresponding supply area is prone to blood supply disturbance after injury.¹² Yue *et al.* found SANFH may cause miRNA changes in femoral head bone microvascular endothelial cells and that miR-132-3p and miR-335 may have important roles in SANFH development.¹³ Our recent research indicated that dexamethasone promoted the production of reactive oxygen species, which increased apoptosis through activation of autophagy and endoplasmic reticulum stress in MC3T3-E1 cells.² In summary, the vascular lesion

of the femoral head caused by steroid is the main cause of SANFH.

VEC Autophagy Related to SANFH

Steroid can damage the blood supply of the femoral head, which can lead to cell ischemia and hypoxia. Stimulation of cells by an extracellular steroid for a long time or in large quantities may cause extensive organelle damage and cellular recycling disorders. Liao *et al.*¹⁴ found that pravastatin can improve dexamethasone-induced avascular necrosis of the femoral head by activating endothelial progenitor cells autophagy, thereby reducing the risk of ONFH and inhibiting the rapid development of autophagy in necrosis. Zhu *et al.*¹⁵ found that the activation of autophagy may be a protective mechanism against apoptosis induced by dexamethasone. Parathyroid steroid (PTH) has a protective effect on SANFH by enhancing autophagy. Other studies have shown that low doses of steroid can cause autophagy, while high doses can lead to apoptosis.¹⁶ Liu *et al.*¹⁷ found that *Herba Epimedii* may have a preventive and therapeutic effect on SANFH by increasing bone mineral density and reducing the autophagy-related genes expression. Other studies have found that in dexamethasone-induced osteoblast injury *in vitro*, autophagy is overactivated and inhibition of autophagy can reduce dexamethasone-induced cell injury.¹⁸ These results show that the relationship between autophagy and SANFH can be ambiguous, which is why researchers contest the role of autophagy in SANFH. Here, observation of four VEC culture moments (12, 24, 48 and 72 h) did not reveal a large-scale enhancement of autophagy in the MP group, but showed that the autophagosome number in this

group was lower than in the Ctrl group. We speculate that this may be related to the rapid autophagy process.

Methylprednisolone May Inhibit the Repair Effect of Autophagy on Damaged VEC

Many experiments have shown that steroid directly damage endothelial cells, leading to vasoconstriction, coagulation, fibrinolysis and femoral head thrombosis, thereby reducing trabecular blood circulation and eventually leading to ONFH.¹⁹ However, steroid-induced vascular injury mechanism is still unclear. Remarkably, 6-keto-PGF1 α may indirectly reflect VEC function as one of the markers of vascular injury.^{20,21} PI3K/Akt/mTOR is an important pathway to regulate autophagy, inhibiting it. On the other hand, inhibition of this pathway can induce autophagy.²² In this study, we found that, compared to the Ctrl group, mRNA expression of PI3K, Akt and mTOR were higher in the MP group, while the mRNA expression of Beclin1 and MAP1LC3 were lower. These data indicates that methylprednisolone can become an inhibitory factor of autophagy after inducing it. The level of VEC autophagy in the MP group was lower than that of the control group in the four evaluated VEC culture time points. In addition, 6-keto-PGF1 α level in the MP group was higher than in the Ctrl group, indicating that methylprednisolone can lead to VEC damage. Besides, in the MP group, with the increase of methylprednisolone action time, PI3K, Akt and mTOR expression and 6-keto-PGF1 α content increased, while Beclin1 and MAP1LC3 expression decreased. This indicates that the longer the methylprednisolone action the lower the physiological autophagy level in VEC and the more serious VEC damage. Methylprednisolone may inhibit the repair effect of autophagy on damaged VEC, which may be one of the reasons for SANFH.

mTOR-shRNA Had Repair Effect on VEC

RNA interference can effectively and specifically silence target genes expression.²³ Zhai *et al.*²⁴ found that after mTOR was silenced by mTOR-siRNA, the expression levels of autophagy-related genes or proteins Beclin 1, LC3-II, Atg5-Atg12 and typical autophagosomes in macrophages increased significantly. Here, we observed that the expression of mRNA of mTOR, PI3K and Akt in MP + shmTOR group was lower than in MP group, indicating that mTOR was

inhibited and PI3K/Akt/mTOR signaling pathway was blocked. At the same time, the mRNA expression of Beclin1 and MAP1LC3 were high in the MP + shmTOR group, indicating an enhancing in autophagy. In addition, it was observed that 6-keto-PGF1 α content was lower in this group compared to the MP group, indicating that VEC damage under the action of mTOR-shRNA was lower than in the MP group. The study also found that the PI3K, Akt, mTOR, Beclin1, MAP1LC3 and 6-keto-PGF1 α levels, and autophagosome number in the mTOR-shRNA group was intermediate between the Ctrl and MP groups. These data suggest that although mTOR-shRNA had a certain repair effect on VEC, compared to the Ctrl group, its autophagy activity was still lower than that of the control group and some damaged cells could not be repaired.

Limitations

However, our study still has limitations, it is worth mentioning that autophagy is a dynamic process and here we observed autophagy only at four time points through *in vitro* experiments. We will continue to refine our experimental and research work to confirm and deepen the results observed in our current work.

Conclusion

In summary, we found that abnormal VEC autophagy is associated with SANFH and VEC autophagy can be regulated by PI3K/ Akt/mTOR signaling pathway. In addition, we also observed that mTOR-shRNA can repair VEC through this signaling pathway. This is the first time that a relationship between VEC, autophagy and PI3K/ Akt/mTOR signaling pathway is observed in the main blood vessels of the femoral head in SANFH.

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