High-carbohydrate, High-fat Diet-induced Hyperlipidemia Hampers the Differentiation Balance of Bone Marrow Mesenchymal Stem Cells by Suppressing Autophagy via the AMPK/ mTOR Pathway in Rat Models

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Research

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Abstract

Background: Disorders of bone metabolism caused by hyperlipidemia is not conducive to osseointegration of implants. Autophagy, an evolutionarily conserved, lysosomal-mediated degradation process, is indispensable for bone homeostasis, its effects on hyperlipidemia-induced osteoporosis remain to be elucidated. The objective of this study was to determine whether autophagy affects bone metabolism and implant osseointegration through regulating the function of bone marrow mesenchymal stem cells (BMMSCs) in rats with hyperlipidemia and to confirm signaling pathway involved in the regulation of autophagy.

Methods: Hyperlipidemia models were established through a long-term high-carbohydrate, high-fat diet in 6-week-old male Sprague-Dawley rats. The impact of hyperlipidemia on bone metabolism and early osseointegration of implants was explored by the methods including serum biochemical detection, micro-computed tomography and bone morphology detection. Biological properties and autophagy levels of BMMSCs were also determined. Further, we determined if autophagy was involved in bone metabolism changes resulting from high-fat diet by focusing on the lineage differentiation of BMMSCs. The signaling pathway involved in the regulation of autophagy was also explored.

Results: The high-carbohydrate, high-fat diet (HCHF) was given to the rats for seven months aggravated bone loss in the cancellous bone and reduced osseointegration of implants. BMMSCs from hyperlipidemia rats exhibited decreased osteogenesis, increased adipogenesis and decreased autophagic activity compared with regular diet (RD) BMMSCs. Rapamycin treatment restored the impaired osteogenic differentiation and inhibited the adipogenic differentiation of HCHF-BMMSCs through the activation of autophagy. Further, AMPK/mTOR signaling pathways was related to the impairment of autophagy of HCHF-BMMSCs.

Conclusions: Our data indicate that long-term high-carbohydrate, high-fat diet-induced hyperlipidemia hampers the differentiation balance of bone marrow mesenchymal stem cells by suppressing autophagy via the AMPK/ mTOR pathway, which ultimately led to aggravated bone loss in the cancellous bone and reduced osseointegration of implants in rat models.

Background

High consumption of high-carbohydrate, high-fat diets links to the development of obesity and hyperlipidemia. Hyperlipidemia is a lipid abnormality characterized by elevated serum cholesterol and triglycerides. Multiple clinical and animal studies suggested that hyperlipidemia and osteoporosis were intimately interrelated [1–3]. Low bone mineral density (BMD) were found in postmenopausal women with high serum total cholesterol (TC). Moreover, statins, a class of cholesterol-lowering drugs, have an anabolic effect on bone [4]. The disorders of bone metabolism caused by hyperlipidemia is not conducive to dental implant therapy since the bone quantity, quality and healing potential are of vital importance in osseointegration [5, 6]. A retrospective study carried out by Tirone et al. which included 227 patient's bone

bonding after 6 months of implant treatment found that hypercholesterolemia could affect the osteogenic effect of bone graft materials [7].

Originating from the mesoderm, BMMSCs are fibroblasts with self-renewal ability and multi-differentiation potential that give rise to both osteoblasts and adipocytes, which is of importance to bone homeostasis [8–9]. As the main source of osteoblasts, BMMSCs gradually differentiate into osteoblasts through a series of signal pathways, and play important roles in bone regeneration and implant osseointegration [10, 11]. Increasing studies have indicated that the abnormal deposition of lipid in the bone space can change the differentiation potential of BMMSCs, which favor of adipogenesis overwhelming osteogenesis in BMMSCs and result in a decrease in bone volume [12, 13]. Diversified external factors and signaling processes dictating the reciprocal regulation between adipocytes and osteoblasts during MSC differentiation and the ultimate control of the adipo-osteogenic equilibrium.

Autophagy is an evolutionarily conserved, lysosomal-mediated degradation process, in which cytoplasmic materials are degraded for reclamation and recycling [14]. Defects in autophagy homeostasis are associated with metabolic disorders, including obesity, diabetes and atherosclerosis, and possess tissue specific [15]. In HFD-induced obese mice, autophagy activity is suppressed in the skeletal muscle and liver [16]. Current, defects in autophagy homeostasis are implicated in the onset of osteoporosis [17–19]. Remarkable bone loss was found in osteoblast-specific autophagy-related gene7 (*Atg7)* conditional knockout mice [20]. The abnormal differentiation of BMMSCs in aging and estrogen deficient osteoporosis mice is related to the decrease of autophagy activity, and the regulation of autophagy can partially reverse the abnormal differentiation balance of BMMSCs [21, 22]. The relationship between autophagy and pathogenesis of osteoporosis induced by high-carbohydrate, high-fat diet-associated hyperlipidemia has not been clearly illustrated.

The objective of this study was to examine the mechanism of high-carbohydrate, high-fat diets-induced-hyperlipidemia on bone metabolism and early osseointegration of implants in rats by focusing autophagy and the lineage differentiation of BMMSCs. Further, signaling pathway involved in the regulation of autophagy was also explored.

Materials And Methods

Animals

40 male Sprague-Dawley rats (140–160 g) were purchased from SPF Animal Research Biotechnology Co., Ltd. (Beijing, China) and maintained in animal facilities as per the protocols approved by the Animal Research Ethical Committee of Shandong University (NO.20190203). After one week of adaptation, rats were randomly allocated to two groups (n = 20). The regular diet group received standard diet, whereas the high-carbohydrate, high-fat diet group received standard chow with addition of 1% cholesterol, 10% lard, 5% yolk powder, 10% sucrose and 0.3% sodium tauroglycocholate. After 24 weeks, serum levels of TC, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride

(TG) of rats were measured using automatic biochemical analyzer (Cobas8000, Switzerland). The rats with dyslipidemia in the control group and the rats failing to meet hyperlipidemic criteria in the experimental group were excluded, which left 18 rats for both the two groups. Then, the pure titanium implant was implanted into the left femur of the rats in the two groups, and the other side was left untreated. The rats were sacrificed after 4 weeks by an injection of sodium pentobarbital intraperitoneally after the implantation to obtain rat serum, followed by relevant detections for the left and right femurs. The right femora were fixed with 4% paraformaldehyde and used to detect bone microstructure.

Microcomputed tomography

The left and right metaphysis of the distal femur of RD and HCHF rats were fixed with 4% paraformaldehyde for 24 h, and then scanned using microcomputed tomography (Scanco Medical AG, Switzerland) at as high as 14.8 μm resolution. The left trabecular of scan started at proximately 1.5 mm proximal to the growth plate of distal femur and with the extension of 5.5 mm. A series of horizontal gray images were generated to reconstruct the three-dimensional (3D) images. The bone-related microarchitectural parameters were evaluated by V6.5-3 software. The cancellous bone microarchitectural parameters, including BMD, bone volume against total volume (BV/ TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), and trabecular separation (Tb. Sp) were measured. Cortical quality parameters including cortical thickness (Ct. Th) and cortical bone area (Ct. Ar), were also measured in the middle area of the femora. After scanning, the 200um cylinder region around the implant of the right femur was set as the region of interest (ROI), and the parameters of trabecula in ROI were analyzed with v6.5-3 software: BV/TV, Tb. Th, TB. N and Tb. Sp.

Histological analysis

Femurs were placed in 4% formalin fixed, decalcified with 10% neutral solution of ethylenediaminetetraacetic acid for 8 weeks. The paraffin-embedded specimens stained with hematoxylin and eosin solutions (Solarbio, China) after sliced into 5-μm-thick sections longitudinally. The microstructure of the femur and the bone around the implants of the two groups were observed and photographed under the light microscope.

BMMSCs isolation and culture

30 males of 6-week-old SD rats were randomly divided into an experimental group and a control group, which included 15 rats for each group. Similarly, the rats in the control group were fed with regular diets, and those in the experimental group were fed high-carbohydrate, high-fat diets. After 24 weeks, the rats with dyslipidemia in the control group and the rats failing to meet hyperlipidemic criteria in the experimental group were excluded, with 12 left in the experimental group and 14 in the control group. The left rats in the two groups were fed continuously for 4 weeks and sacrificed on 28th week. BMMSCs were isolated and cultured using the whole bone marrow adherence method. In brief, BMMSCs were isolated from RD and HFD rats by flushing the femurs and tibias with α-Modified Eagle's Medium (α-MEM, HyClone, USA), containing 20% fetal bovine serum (FBS, HyClone, USA) and 1% penicillin/streptomycin

(Gibco, USA), and then cultured in 10-cm culture dishes under a 5% humidified CO2 atmosphere at 37 °C. The medium was refreshed every two days.

Immunophenotypic Characterization of BMMSCs

To characterize immunophenotypes, BMMSCs isolated from RD and HFD rats were analyzed by flow cytometry. Briefly, cells were trypsinized and incubated with CD90, CD29, CD44, CD45 and CD11b for 30 minutes. Further, cells were analyzed by flow cytometry after unbound antibodies were removed.

colony forming unit (CFU) analysis

To assess the self-renewal capacity of BMMSCs, 1000 primary cultured BMMSCs from control and experimental groups were seeded in 10-cm dishes. After 7 days of culture, the cells were fixed with 4% paraformaldehyde, and then 0.1% toluidine blue was applied to stain the colonies. Further, colonies of more than 50 cells were counted and analyzed.

Osteogenic induction and alizarin red staining

The osteo-differentiation of BMMSCs was induced using osteogenic induction medium (a-MEM, 10% FBS, 50 μg/ml ascorbic acid, 10 mM dexamethasone, and 10 mM β- glycerophosphate). After 21 days of culture, BMMSCs were stained with 2% alizarin red S, pH 4.2 after fixed with 10% paraformaldehyd. For quantification, cells were incubated with 10% cetylpyridinium chloride, then the absorbance was read at 562 nm.

Adipogenic induction and the Oil red staining

Adipogenic lineage differentiation of BMMSCs was induced using adipocytic induction medium (a-MEM, 10% FBS, 1uM dexamethasone, 10ug/ml insulin, 0.5 mM isobutyl methylxanthine and 0.2 mM indomethacin (sigma, USA)) for 14 days. Cells were fixed with 10% paraformaldehyde, and stained for 30 min with 0.5% Oil Red O solutions. For quantification, the dye was eluted with isopropanol, and the absorbance was read at 490 nm.

Transmission electron microscope (TEM)

BMMSCs were fixed overnight in 2.5% glutaraldehyde at 4℃. The specimens were prepared according to the described process [16] and samples were detected using JEM 1400 transmission electron microscope (JEOL, Inc, USA).

Adenoviral infection

The AdPlus-mCherry-GFP-LC3B adenovirus (Beyotime, China) was used to transfect BMMSCs to indicate autophagy flux. In short, cells were incubated with AdPlus-mCherry-GFP-LC3B adenovirus at a multiplicity of infection of 40 when reached 50% confluence. The medium was refreshed after 24 h with fresh complete medium. The cells were cultured for a further 24 h. The mRFP-GFP-LC3 dot formation was captured by confocal laser scanning microscope (CLSM, Olympus, Japan).

Autophagy regulation

To control autophagy of BMMSCs, the cells were treated with rapamycin (100 nM) (TargetMol, China) to induce autophagy, and 3-methyladenine (2 mM) (TargetMol, China) was used to inhibit autophagy before osteogenic or adipogenic differentiation induction, respectively. Besides, AICAR (10umol/L) (TargetMol, China) were used to activate AMPK of HFD-BMMSCs to explore the related signaling pathways.

qRT-PCR

Total RNA was extracted from BMMSCs using RNAiso Plus (TaKaRa, China) and was reversely transcribed into cDNA. The target genes expression was quantified by qRT-PCR. All genes were normalized to *β-actin* and analyzed with the $2^{-\triangle\triangle CT}$ method. The primer sequences are listed in Table 1.

Table 1

Primer sequences of rat gene.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GAAGTGTGACGTTGACATCCG	GAAGTGTGACGTTGACATCCG
Alp	TTGACCTCCTCGGAAGACACTCTG	CGCCTGGTAGTTGTTGTGAGCATAG
Runx2	ACTTCCTGTGCTCGGTGCT	GACGGTTATGGTCAAGGTGAA
Col1	GAGAGCATGACCGATGGATT	CCTTCTTGAGGTTGCCAGTC
Lc3	GAGTGGAAGATGTCCGGCTC	CCAGGAGGAAGAAGGCTTGG
Beclin1	TCACTCTGATCGGGGAGGCAT	TCGCCCTCATTCATTTGCTCC
P62	GCTGCTCTCTTCAGGCTTACAG	CCTGCTTCACAGTAGACGAAAG
Ppar-γ	CTGCGTCCCCGCCTTAT	TTCAATCGGATGGTTCTTCG

Western blot

Total protein from BMMSCs were extracted by RIPA buffer. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated with specific primary antibody overnight at 4°C after blocked in 5% dehydrated milk. The membranes were incubated in secondary antibody for 1 h. Obtained bands were visualized with an ECL kit (Millipore, USA). Further, the software ImageJ (version 1.8.0, National Institutes of Health, USA) was used to quantify the band intensity, and the signal of each target band was normalized to GAPDH band. Primary antibodies were as follows: Runx2, PPAR-γ, COL1, AKT, p-AKT, AMPK and p-AMPK (Cell Signaling Technology, USA); BECLIN-1 (Santa Cruz, USA), LC3, P62, ALP, mTOR and p-mTOR (Abcam, UK).

Statistical analysis

All data are expressed as mean ± standard deviation for 3 independent experiments. Student's t-test was used to evaluate the differences between the two groups, whereas, $P < 0.05$ was considered as significant.

Results

Femoral bone mass and new bone around the implants were reduced in high-carbohydrate, high-fat diets-induced hyperlipidemia rats

We successfully constructed the rat model of hyperlipidemia, which was confirmed by elevated levels of TC and LDL-C, and reduced level of HDL-C (Fig. 1A). Compared to the control group, the biochemical markers P1NP related to bone formation decreased significantly, while CTX-1 related to bone resorption increased significantly in the hyperlipidemia group (Fig. 1B). Our results indicated that long-term high-fat diet led to cancellous bone loss at distal femur characterized by reduced cancellous BMD, BV/TV, Tb.N, and increased Tb.Sp. (Fig. 1C, D). However, the cortical parameters were observed to be not obviously changed (Fig. 1E, F); Hyperlipidemia led to the decrease of new bone around implants characterized by reduced BV/TV, Tb.Th, Tb.N and TB.Sp (Fig. 1G, H). Moreover, HE staining results showed that incomplete trabecular bone structures, increased marrow adiposity within the bone marrow cavity (Fig. 1I) and inafiltration of adipocytes in the trabecular spaces of new bone tissue around the implants were seen in the femur of hyperlipidemic rats (Fig. 1J).

Fig. 1 Femoral bone mass and new bone around the implants were reduced in high-fat diet-induced hyperlipidemia rats. (A) Serum levels of TC, TG, LDL -C, HDL-C. (B) Serum biochemical test. (C) Representative micro-CT images of distal femur. (D) Trabecular bone parameters: BMD, BV / TV, Tb. N, Tb. Sp, and Tb. Th. (E) Representative micro-CT images of the cortical bone in the middle area of the femora. (F) Cortical bone parameters: Ct.Th and Ct.Ar. (G) Representative micro-CT images of the femoral implants in rats. (H) Parameters of new bone around implants: BV/TV, Tb.Th, Tb.N and Tb.Sp .(I)Images of distal humerus stained with HE. (J) Bone microtructure around the femoral implant stained with HE. The black arrows indicate adipocytes. Data are reported as means ±SD. *$P < 0.05$ Vs. RD group. RD: regulated diet. HCHF: high-carbohydrate, high-fat diet.

BMMSCs derived from HFD rats displayed impaired osteogenic differentiation capacity and enhanced adipogenic differentiation capacity

BMMSCs derived from RD and HCHF rats were used to analyze the cell biological properties. As shown in Fig. 2A, the primary BMMSCs derived from RD and HCHF rats were fusiform or triangular, mixed with round, highlighted and unattached hybrid cells. The self-renewal capacity of HCHF-BMMSCs were significantly reduced than RD-BMMSCs as indicated by CFU analysis (Fig. 2B). BMMSCs of both origins were characterized by positive expression of CD90, CD29 and CD44 while negative expression of the hematopoietic markers CD45 and CD11b (Fig. 2C). HCHF-BMMSCs exhibited aberrant differentiation commitment. Significant reduction of osteogenic differentiation ability was observed in HCHF-BMMSCs. HCHF-BMMSCs formed fewer alizarin red-positive nodules, which was in line with the decreased expression of Runx2, ALP and Col1 at both the mRNA and protein levels (Fig. 2D, F). Compared with RD-

BMMSCs, HCHF-BMMSCs showed increased adipogenic differentiation and this was evident from the Oil red staining (Fig. 2E). PPAR-γ is the adipocyte-specific factor essential for adipocyte differentiation. From our results it was confirmed that the expression of PPAR-γ mRNA and protein were increased in HCHF-BMMSCs (Fig. 2G).

Fig. 2 BMMSCs derived from high-fat diet rats displayed with decreased self-renewal capacity, osteogenic differentiation capacity and increased adipogenic differentiation capacity. (A) Cell morphology (scale bar = 200μm). (B) colony forming unit analysis. (C) Immunophenotypic Characterization of BMMSCs analyzed by flow cytometry. (D) Cytological staining of alizarin red to detect matrix mineralization in BMMSCs culture induced for osteogenesis for 21 d (scale bar = 200μm), and quantitative analysis performed using cetylpyridinium chloride. (E) Cytological staining of oil red O to detect fat droplets in the cytoplasm of BMMSCs induced for adipogenic differentiation for 14 d (scale bar = 50μm), and quantitative analysis performed using isopropanol. (F) q RT-PCR and Western blot were used to assess the mRNA and protein levels of Runx2, ALP, Col 1 in BMMSCs culture induced for osteogenesis for 14 d. (G) q RT-PCR and Western blot were used to assess the mRNA and protein levels of PPAR-γ in BMMSCs culture induced for adipogenic for 14 d. Data are reported as mean ± SD. *P< 0.05 Vs. control group. **P< 0.01 Vs. control group. RD: regulated diet. HCHF: high-carbohydrate, high-fat diet.

Autophagy levels declined in BMMSCs derived from high-carbohydrate, high-fat diet

Research shows that autophagy occupies important roles in bone homeostasis. In this study, a series of methods were adopted to examine autophagy levels of RD-BMMSCs and HCHF-BMMSCs. During the study, we measured the expressions of Beclin1 and microtubuler associated protein 1 light chain 3 (LC3), which is of key importance in the initiation and maturation of autophagosome, and P62, an indicator of autophagosomal degradation. Impaired autophagy was found in HCHF-BMMSCs. The gene expression levels of *Beclin1* and *Lc3* decreased, while *P62* increased in HCHF-BMMSCs (Fig. 3A). Decreased expression of Beclin1 and reduced LC3II/LC3I ratio, as well as accumulated P62 were observed in HCHF-BMMSCs by Western blot (Fig. 3B). Further, the vacuoles labeled with mCherry-GFP-LC3B were detected by confocal laser scanning microscope (LSCM) to examine the status of autophagic flux. Colocalization of RFP-LC3 and GFP-LC3 dots (yellow) were lower in HFD-BMMSCs (Fig. 3C). Autophagosomes are spherical structures with double-layer membranes enclosing damaged or unnecessary organelles. Autophagosomes can be identified by TEM, and we found that HCHF-BMMSCs possessed fewer autophagosomes than RD-BMMSCs (Fig. 3D).

Fig. 3 Autophagy level declined in BMMSCs from high-fat diet rats. Beclin1, Lc3 and P62 mRNA were examined by qPCR. (A) Beclin1, Lc3 and P62 mRNA were examined by qPCR.; (B) The protein level of Beclin1, LC3 and P62 in RD- and HFD-BMMSCs were examined by Western blot. (C) LSCM Images of BMMSCs transfected with AdPlus-mCherry-GFP-LC3B adenovirus. The fusion dot number (yellow) indicated the autophagy level (scale bar =20μm). (D) Autophagosomes of RD- and HFD-BMMSCs detected by TEM. Top row: scale bar = 5μm. Bottom row: scale bar = 1μm. Arrows indicated the typical

autophagosomes. Data are reported as mean ± SD. *P < 0.05 Vs. RD group. RD: regulated diet. HCHF: high-carbohydrate, high-fat diet.

Autophagy was essential in maintaining the differentiation balance of BMMSCs derived from high-fat diet rats

Previous studies suggest that the autophagy is involved in regulation of cellular differentiation. HCHF-BMMSCs manifested degraded osteogenic potential and impaired autophagy activity. Therefore, we concentrated on the autophagy to explore the probable cause of the anomalous differentiation of HCHF-BMMSCs. The rapamycin promoted osteogenic differentiation, but inhibited the adipogenic differentiation of HCHF-BMMSCs through activation of autophagy. The increased expression of RUNX2 and decreased PPAR-γ were further confirmed by our results (Fig. 3A, B). The 3-MA, an autophagy inhibitor, inhibited the osteogenic differentiation, but increased the adipogenic differentiation of RD-BMMSCs, as shown by alizarin red and oil red staining. The decreased expression of RUNX2 but elevated expression of PPAR-γ was further confirmed by the results shown in Fig. 3C, D. These results indicated that autophagy has an essential role in differentiation of BMMSCs, and activating autophagy with rapamycin partially restored the anomalous differentiation of HFD-BMMSCs.

Fig. 4 Autophagy was essential in maintaining the differentiation balance of BMMSCs derived from high-fat diet rats. (**A**) Western blot was performed to examine expressions of LC3, P62 and Runx2 protein level and alizarin red staining was performed to detect mineralized nodules formed in HCHF-BMMSCs cultured in osteo-differentiation medium in the presence of rapamycin. (B) Western blot was performed to examine expressions of LC3, P62 and PPAR-γ protein level and Oil Red O staining was performed to detect lipid droplet formed in HCHF-BMMSCs cultured in adipo-differentiation medium in the presence of rapamycin. (C) Western blot was performed to examine expressions of LC3, P62 and Runx2 protein level and alizarin red staining was performed to detect mineralized nodules formed in RD-BMMSCs cultured in osteo-differentiation medium in the presence of 3-MA. (D) Western blot was performed to examine expressions of LC3, P62 and PPAR-γ protein level and Oil Red O staining was performed to detect lipid droplet formed in RD-BMMSCs cultured in adipo-differentiation medium in the presence of 3-MA. Oil Red O staining (Scale bar =50μm). RD: regulated diet. HCHF: high-carbohydrate, high-fat diet.

AMPK/mTOR signaling pathway was involved in the regulation of autophagy and osteogenesis of BMMSCs derived from high-carbohydrate, high-fat diet

As shown in Fig. 5A, the BMMSCs in the HCHF group were found to have increased p-mTOR/mTOR, insignificantly changed p-Akt/Akt, and decreased p-AMPK/AMPK compared with the rats in the control group. These results suggested that AMPK/mTOR signaling pathway was presumably associated with the impaired autophagy of BMMSCs after long-time exposure to the high-carbohydrate, high-fat diet. As the first found AMPK activator, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) is widely used in AMPK and related experimental research. The BMMSCs in the HCHF group after treatment with AICAR demonstrated increased p-AMPK/AMPK, decreased p-mTOR/mTOR, enhanced autophagy activity which was evident from the increased LC3II/LC3I ratio and reduced P62 expression by Western

blot (Fig. 5B). We also observed enhanced osteogenic ability which was evident from increased expression of Runx2 and more alizarin red-positive nodules (Fig. 5C). These results indicated that AMPK/mTOR signaling pathway was related to the impairment of autophagy and osteogenesis of BMMSCs derived from high-carbohydrate, high-fat diet.

Fig. 5. AMPK/mTOR signaling pathway was related to the impairment of autophagy and osteogenesis of BMMSCs derived from high-fat diet rats. (A) Western blot was performed to examine expressions of p-mTOR, mTOR, p-Akt, Akt, p-AMPK, AMPK, LC3 and P62 protein in RD- and HFD-BMMSCs. (B) Western blot was performed to examine expressions of p-mTOR, mTOR, p-AMPK, AMPK, LC3 and P62 protein in HCHF-BMMSCs in the presence of AICAR. (C) Western blot was performed to examine expression of Runx2 protein and alizarin red staining was performed to detect mineralized nodules formed in HCHF-BMMSCs cultured in osteo-differentiation medium in the presence of AICAR. Data are reported as mean ± SD. *P < 0.05 Vs. RD group. RD: regulated diet. HCHF: high-carbohydrate, high-fat diet.

Discussion

Long-time high-carbohydrate, high-fat diets-induced hyperlipidemia exert adverse effects on bone metabolism and implant osseointegration in rats, however the underlaying molecular mechanism is still poorly understood. The present study determined the effects of hyperlipidemia on bone metabolism and implant osseointegration through the new perspectives of the AMPK/mTOR signaling pathway, autophagy and the lineage differentiation of BMMSCs. In the present study, hyperlipidemia reduced the basic autophagy activity of BMMSCs in rats by inhibiting the AMPK/mTOR pathway, and the reduced autophagy level further led to abnormal differentiation balance of BMMSCs, which ultimately led to aggravated bone loss in the cancellous bone and reduced osseointegration of implants.

The high incidence rate of hyperlipidemia and its adverse effects on bone metabolism have attracted profound attention. This study simulated the diet habits of modern people through long-term high-carbohydrate, high-fat diet feeding and led to construction of successful rat model for hyperlipidemia. The present study, as previously reported [2, 23], validated that the deleterious skeletal changes were found in cancellous bone, but not in cortical bone of hyperlipidemia rats, which may be due to the compact and solid structure of cortical bone and the lower remodeling activity than that of honeycomb cancellous bone. An in vivo study demonstrated that hyperlipidemia significantly increased implant loss and decreased the formation of the bone-to-implant interface in the mouse femur [24]. In this study, hyperlipidemia affected the osseointegration of implants in rats, resulting in the decreased new bone around the implants.

The analyzed histomorphometrics of bone in the proximal femur presented a significant decline in trabecular bone volume, along with an increase in bone marrow adiposity compared with those of the RD groups. Moreover, we observed infiltration of adipocytes in the trabecular spaces of new bone tissue around the implants. Plenty of studies indicate that bone marrow fat is implicated in metabolism and bone health. The abnormally enhanced adipogenic differentiation of BMMSCs is closely associated with

the fat accumulation in bone marrow and bone loss during osteoporosis [25]. Previous studies suggested that high-fat diet suppressed bone formation by hampering the differentiation of osteoblast progenitor cells and facilitating the adipogenesis of BMMSCs [13]. The obesity promotes an inflammatory microenvironment in bone marrow that commits BMMSCs to adipogenesis [26]. In this study, BMMSCs derived from hyperlipidemia rats exhibited decreased osteogenesis and increased adipogenesis, might be partly responsible for the abnormal bone metabolism and the poor osseointegration of implants. However, the underlying mechanism of abnormal lineage differentiation of HCHF-BMMSCs is still ambiguous and demands more precise explication.

Previous studies found that autophagy is involved in maintaining the stemness and multipotential differentiation of stem cells and plays important roles in the onset and progression of pathological osteoporosis [27]. Degenerative autophagy and osteogenic ability of BMMSCs derived from the vertebral body in the elderly patients with osteoporosis and aged mice can be restored by autophagy activation [21, 18]. In high-fat diet-induced obese mice, impaired autophagy is reported to be the basis of decreased insulin sensitivity [29]. It is well documented that Beclin1, LC3 and P62 are special phenotypic markers of autophagy. As a crucial component of the class III phosphatidylinositide-3-kinases (PI3K) complex, Beclin1 is of key factor in the initiation and maturation of autophagosome. Generally, LC3 exists in the cytoplasm in the LC3-I form under normal conditions, and once autophagy occurs, LC3-I conjugates to phosphatidylethanolamine in autophagosome membrane to form LC3II. Therefore, the ratio of LC3II/LC3I is usually used to reflect the level of autophagy [30]. The P62, sqstm-1, serves as the degradation substrate of autophagy and combines with the membrane type LC3 when autophagy occurs and then it gets degraded, which is the sign of autophagy flow [31]. As a process of adaptive response of cells to the external environment, autophagy level is affected by hormones, inflammatory factors, and early stage of stress in which autophagy level increases. Whereas, when external stimulation persists, autophagy level decreases [32]. To investigate the effect of hyperlipidemia on autophagy level of BMMSCs, a series of methods were adopted. In our study, decreased Beclin1, reduced LC3II/LC3I ratio, accumulated P62, as well as reduced autophagosomes were observed in HCHF-BMMSCs. This demonstrates that the level of basic autophagy in HCHF-BMMSCs reduced, in consistent with the changes of autophagy level of BMMSCs in estrogen deficiency and aging osteoporosis.

It was unclear whether the decreased autophagy level related to the abnormal differentiation balance of BMMSCs derived from hyperlipidemia rats. The rapamycin, a specific suppressor of mammalian target of rapamycin (mTOR), was used as autophagy activator to explore the lineage differentiation of HCHF-BMMSCs [33]. Our results showed that rapamycin improved the autophagy level of HCHF-BMMSCs. At the same time, its osteogenic differentiation ability was enhanced, and its adipogenic differentiation capacity was weakened. Previous studies found that the rapamycin treatment could rescue the impaired differentiation function of BMMSCs and increase bone mass by reactivating autophagy in OVX mice [22]. In addition, autophagy inhibitors have been reported to impair the differentiation of dental pulp-derived MSCs into osteoblasts [34]. 3-MA, a selective PI3K inhibitor, can specifically and effectively blocked the fusion process of lysosome and autophagy [35]. Previous study found that 3-MA could inhibit the osteogenic differentiation and mineralization of BMMSCs [36]. In this study, downregulation of

autophagy by 3-MA, resulted in reduced osteogenic differentiation and enhanced adipogenic differentiation of RD-BMMSCs. This study preliminarily demonstrated that autophagy plays an important role in the balance of differentiation of MSCs, and the reduction of basic autophagy level of MSCs in rats with hyperlipidemia may be one of the potential mechanisms leading to the abnormal differentiation balance of mesenchymal stem cells and decreased bone mass.

Activation of autophagy by rapamycin, a specific inhibitor targeting mTOR, can partially reverse its weakened osteogenic differentiation ability of HCHF-BMMSCs. mTOR, a serine/threonine kinase, participates in the transduction of multiple signal pathways in vivo. mTOR exists in two distinct complexes, namely, mTORC1 and mTORC2, whereas, mTORC1 is mainly related to autophagy [37]. mTORC1/autophagy plays an important role in bone metabolism. Rapamycin can reverse the osteopenia phenotype of myofibrillary 1-deficient mice by inhibiting mTOR to regulate the differentiation direction of BMMSCs [38]. In the present study, the phosphorylation of mTOR in HCHF-BMMSCs was upregulated, which revealed that autophagy might be inhibited through activation of mTOR pathway in BMMSCs derived from hyperlipidemia rats. Autophagy is precisely regulated by a series of complex signaling pathways, among which AMPK/mTOR and PI3K/Akt/mTOR are the two most studied pathways, closely related to mTOR. Adenylate activated protein kinase (AMPK), a serine/threonine kinase, is involved in the regulation of autophagic activity in osteoblast differentiation [39]. PI3K/Akt/mTOR signaling pathway is a widespread signaling pathway in cells, closely related to cell proliferation, differentiation and autophagy, and is a negative regulatory pathway for autophagy [40].

Therefore, we detected the key signal molecules of the two pathways using immunoblotting, and we found that, compared to the control group, the ratio of p-AMPK/AMPK was downregulated, while the change of p-Akt/Akt was not obvious, which suggested that AMPK/mTOR signaling pathway mediated the damaged autophagy in mesenchymal cells under high fat environment. Based on its role in intracellular energy sensor, AMPK is an important regulator of intracellular energy and metabolic balance [41]. Xu et al. [42] found that long-term high-fat diet can reduce the autophagy level of mouse hepatocytes through the p-AMPK/mTOR signaling pathway. AMPK also plays an important role in the physiological activities of bone tissue. Shah et al. [43] found that the bone mass of AMPK 1 knockout mice was significantly reduced compared with the wild-type mice. Studies showed that AMPK could inhibit PPAR-γ by phosphorylation of β-catenin, thus inhibiting the adipogenic differentiation of MSCs in vitro [44]. Changes in metabolic pathways are associated with bone tissue cell differentiation or function, and AMPK, as a cellular energy sensitive factor, is involved in the regulation of autophagy by the mTOR signaling pathway [45]. Moreover, study carried out by Pantovix et al. [46] found that both early mTOR inhibition-mediated autophagy and late activation of Akt/mTOR signaling axis were involved in AMPK controlling osteogenic differentiation of hMSCs. The 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), also known as acadesine, is the first reported AMPK activator, which can combine with the γ subunit of AMPK and mimic the allosteric activation of AMP on AMPK, and is widely used in AMPK related experimental research [47]. In this study, reactivating AMPK by AICAR inhibited the expression of mTOR, activated autophagy and enhanced the osteogenic ability of HCHF-BMMSCs, which

indicated that AMPK/mTOR signaling pathway was related to the impairment of autophagy and osteogenesis of mesenchymal cells in high-fat environment.

Conclusions

In conclusion, this study preliminarily confirmed that long-term high-carbohydrate, high-fat diets induced hyperlipidemia reduced the basic autophagy activity of BMMSCs in rats by inhibiting the AMPK/mTOR pathway, and the reduced autophagy level further led to abnormal differentiation balance of BMMSCs, which ultimately led to aggravated bone loss in the cancellous bone and reduced osseointegration of implants. Our results suggested that autophagy in BMMSCs was one of the potential mechanisms for the abnormal differentiation balance of BMMSCs and might be a new and effective therapeutic target for hyperlipidemia-related bone loss. For future research, there is need of focused efforts specifically should be invested in exploring the role of autophagy in implant osseointegration and osteoporosis resulting from hyperlipidemia by constructing the lentiviral vector-mediated overexpression of autophagy-related genes or controlling the carriers of the genes.

Abbreviations

BMMSCs
bone marrow mesenchymal stem cells; HCHF:high-carbohydrate, high-fat diet; RD:regular diet; BMD:bone mineral density; TC:total cholesterol; *Atg7*:autophagy-related gene7; LDL-C:low-density lipoprotein cholesterol; HDL-C:high-density lipoprotein cholesterol; TG:triglyceride; CTX-1:C-telopeptide of type 1 collagen; P1NP:Procollagen type I N-terminal propeptide; BV/TV:bone volume against total volume; Tb. Th:trabecular thickness; Tb. N:trabecular number; Tb. Sp:trabecular separation; Ct. Th:Cortical quality parameters including cortical thickness; Ct. Ar:cortical bone area; ROI:region of interest; α-MEM:α-Modified Eagle's Medium; FBS:fetal bovine serum; 3-MA:3-methyladenine; CFU analysis:colony forming unit analysis; TEM:Transmission electron microscope; AICAR:5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside; qRT-PCR:Real-time quantitative polymerase chain reaction; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; β-catenin:catenin beta1; ALP:Alkaline phosphatase; RUNX2:Runt-related transcription factor 2; COL1:type I collagen; PPAR-γ:peroxisome proliferators-activated receptorsγ; LC3:microtubulerassociated protein 1 light chain 3; P62:PI3K:class III phosphatidylinositide-3-kinases; AKT:protein kinase B; AMPK:Adenylate activated protein kinase; mTOR:mammalian target of rapamycin

Declarations

Acknowledgments

Not applicable.

Author Contributions

Huiqiang Sun, Xing Liang and Zhenzhen Shang designed the research; Zhenzhen Shang performed research; Ting Zhang, Mengyang Jiang and Xiaojie Yin analyzed the data; Zhenzhen Shang wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Ethics approval

The animal experimental protocol was approved by the Animal Research Ethical Committee of Shandong University (NO.20190203) (Jinan, China).

Consent for publication

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Competing interests

The authors declare no conflict of interest.

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