Asiatic acid improves high-fat-diet-induced osteoporosis in mice via regulating SIRT1/FOXO1 signaling and inhibiting oxidative stress

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Asiatic acid improves high-fat-diet-induced osteoporosis in mice via regulating SIRT1/FOXO1 signaling and inhibiting oxidative stress

Running title: Asiatic acid in osteoporosis

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Abstract

Asiatic acid can attenuate osteoporosis through suppressing adipogenic differentiation and osteoclastic differentiation. Oxidative stress enhances osteoclastic differentiation but represses osteogenic differentiation to promote osteoporosis. However, the role and mechanism of asiatic acid in osteoporosis have not been reported. Firstly, mice were fed with high-fat-diet (HFD) with or without asiatic acid for 16 weeks. Data from an automatic biochemical analyzer showed that HFD induced down-regulation of high-density lipoprotein (HDL) and an increase of serum levels of triglyceride (TG), total cholesterol (TC) and low-density lipoprotein (LDL). However, asiatic acid administration attenuated the decrease of HDL and increase of serum TG, TC and LDL in osteoporotic mice. Secondly, HFD induced high levels of malondialdehyde (MDA) and reactive oxygen species (ROS), low levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in osteoporotic mice. However, the levels of MDA, ROS, SOD and GSH-Px in osteoporotic mice were reversed by asiatic acid administration. (this section is unclear and requires revision) Asiatic acid administration reduced expression of c-telopeptide of type 1 collagen (CTX-1), enhanced alkaline phosphatase (ALP) and procollagen type 1 N-terminal propeptide (P1NP) in HFD-induced osteoporotic mice. (this section is unclear and requires revision) Thirdly, asiatic acid promoted calcium deposition in bone marrow cells and osteogenic differentiation in osteoporotic mice, but decreased ALP in bone marrow cells. Lastly, asiatic acid enhanced SIRT1 and nuclear FOXO1 (Nu-FOXO1) expression, while it reduced Acetyl FOXO1 (Ac-FOXO1) in osteoporotic mice. In
conclusion, asiatic acid might inhibit oxidative stress and promote osteogenic differentiation through activating SIRT1/FOXO1 to attenuate HFD-induced osteoporosis in mice.

Keywords: Asiatic acid, oxidative stress, osteogenic differentiation, SIRT1/FOXO1, osteoporosis

Introduction

Osteoporosis is a common bone metabolic disease that affects the elderly and postmenopausal women with high morbidity and mortality (Ybarz et al., 2011). Advanced therapeutic strategies have been developed to ameliorate osteoporosis. For example, bisphosphonates with the ability to repress bone resorption is used as the first-line pharmacologic treatment for osteoporosis (Diab and Watts, 2012). However, bisphosphonates could induce uncommon side effects, including inflammatory eye disease and fractures of the femur (Janovská, 2012). Risk factors to induce osteoporosis include age, heredity, oestrogen deficiency, and diet (Yedavally-Yellayi et al., 2019). Research has shown that people with high body fat possessed high risk for suffering osteoporosis with ageing (Tomlinson et al., 2019). A high-fat diet (HFD) has been reported to be a negative regulator on bone health, and bone strength was reduced in mice followed by HFD consumption (Devlin et al., 2018). Therefore, strategies to regulate HFD-induced fat and energy metabolism can prevent development of osteoporosis (Carnovali et al., 2018).
Reactive oxygen species accumulation has been shown to be involved in bone metabolism (Sontakke and Tare, 2002). The imbalance in production of antioxidant and reactive oxygen species induces oxidative stress, which participates in the progression of osteoporosis (Abdollahi et al., 2005). Oxidative stress inhibits the differentiation and mineralization of osteoblasts, promotes the differentiation of osteoclasts, and reduces bone mass and bone strength, thus aggravating the occurrence and development of osteoporosis (Abdollahi et al., 2005). Suppression of oxidative stress promotes osteogenic differentiation of bone marrow mesenchymal stromal cells into osteoblasts, and attenuates bone loss during the development of osteoporosis (Yang et al., 2021).

Asiatic acid, a triterpenoid compound extracted from Centella asiatica, has been shown to possess antihyperlipidemia, antidiabetic and anticancer properties (Tomlinson et al., 2019). Asiatic acid also enhanced antioxidative effects and suppressed lipid peroxidation to attenuate streptozotocin-induced diabetes (Ramachandran and Saravanan, 2013), and exerted antioxidative and anti-inflammatory abilities to ameliorate metabolic syndrome in diet-induced rats (Tomlinson et al., 2019). Obesity-related osteoarthritis (Yu et al., 2020) and the adipogenic differentiation of bone marrow stromal cells (Li et al., 2014) were repressed by asiatic acid. Moreover, asiatic acid reduced bone loss (Huang et al., 2019), suppressed osteoclastogenesis and promoted osteogenic differentiation (Tomlinson et al., 2019) in ovariectomized-induced osteoporosis. However, the effects of asiatic acid on HFD-induced osteoporosis, as well as osteoporosis-associated
oxidative stress, remain elusive.

Activation of SIRT1/FOXO1 has been shown to be involved in redox balance and osteogenic differentiation during development of osteoporosis (Jiang et al., 2020). Asiatic acid regulated the activation of the SIRT1 pathway (Qian et al., 2018). This study speculated that asiatic acid might suppress oxidative stress and promote osteoclastic differentiation in HFD-induced osteoporosis mice model through regulation of SIRT1/FOXO1 pathway.

Materials and method

Animal experiments

Twenty-four male C57BL / 6 mice (4 weeks old, 10-15 g weight) were purchased from Vital River Laboratory Animal Technology Co Ltd. (Beijing, China), and maintained in cages with 12-h light/dark cycle and constant temperature (24 - 26 °C). An HFD-induced osteoporosis mouse model was established according to previous research (Chen et al., 2019). The mice were randomly assigned to four groups: control group with normal control diet; HFD group with high fat diet (carbohydrates, 20.3%; fat, 61.6%; protein, 18.1%); HFD with 25 mg/kg asiatic acid and HFD with 50 mg/kg asiatic acid. Asiatic acid was prepared according to previous work (Hong et al., 2020). Mice in HFD with asiatic acid groups were orally administered with asiatic acid once per day, and mice in the control and HFD group received vehicle as control. Mice were fed for 16 weeks, and then the serum and bone samples were collected from the mice. The experiments were approved by Affiliated Hospital of Guangdong Medical
University and in accordance with National Institutes of Health Laboratory Animal Care and Use Guidelines.

Serum samples

Mice were fasted for 12 hours, and then euthanized by carbon dioxide inhalation after 16 weeks post specified diet feeding. Blood samples were collected, and then centrifuged at 3000 g for 10 minutes to harvest the serum samples. Serum levels of TG, TC, LDL and HDL were measured by an automatic biochemical analyzer (Beckman Coulter, Inc., Brea, CA, USA). Levels of MDA, SOD, GSH-Px, ROS, ALP, P1NP and CTX-1 were measured by using commercial ELISA kits (Cusabio, Wuhan, China).

Bone histology analysis

The right femurs were collected from the mice, and fixed in 4% paraformaldehyde. The femurs were decalcified in 10% ethylenediaminetetraacetic acid, and embedded in paraffin. The femurs were cut into longitudinal sections with 5-µm thickness, which were then stained with haematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). The sections were observed under a light microscope (Zeiss, Zeppelinstrasse, Germany) at 400× magnification. Trabecular area was measured using Image-Pro Plus 6.0.
Calcein double labelling

Mice were intraperitoneally injected with 0.5 mg calcein (Sigma-Aldrich) twice with the first time at tenth day before euthanasia and the second time at 2nd day before euthanasia. The longitudinal sections of femora were then performed with calcein double labelling. The sections were observed under fluorescence microscope (Zeiss), and mineral apposition rate (MAR), bone formation rate (BFR), trabecular bone volume (BV), trabecular bone surface (BS) and total tissue area (TV) were evaluated using Image-Pro Plus 6.0.

Bone marrow cell culture

The femora were excised from the mice, and the epiphyses were cut off from the femora after sixteen weeks post the specified diet feeding. The marrow was flushed out, and the bone marrow cells were cultured in α-MEM containing 10 % fetal bovine serum, 50 µg/ml ascorbic acid, 10 mM-β-glycerophosphate and 100 nM-dexamethasone at 37 °C. Cells were cultured in an incubator. Twelve days post culture, cells were fixed in 4% paraformaldehyde, and then stained in oil red solution (Sigma-Aldrich) before observation under a microscope. After cells were cultured for fourteen days, ALP activity was determined at 405 nm with an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Twenty-one days later, the cultured cells were fixed in 4 % formaldehyde, and stained with Alizarin Red-S (40 mM; Sigma-Aldrich). Cells were observed under the microscope at 200× magnification.
Western blot

Proteins were extracted from the bone marrow cells by RIPA lysis buffer (Sigma-Aldrich), and the concentration was quantified by QuantiPro™ BCA Assay Kit (Sigma-Aldrich). The proteins were separated by SDS-PAGE and then transferred onto PVDF membrane (Millipore, Billerica, MA, USA). The membrane was incubated with blocking solution (TBST buffer containing 5% skimmed milk powder and 0.05% Tween 20), and then probed with primary antibodies: anti-Collagen-1 and anti-RUNX2 (1:2000; Abcam, Cambridge, UK), anti-osteocalcin and anti-GAPDH (1:3000; Abcam), anti-SIRT1 and anti-Histone H3 (1:3500; Abcam), anti-Ac-FOXO1 and anti-Nu-FOXO1 (1:4000; Abcam). The membranes were then incubated with horseradish peroxidase-conjugated anti-IgG secondary antibody (1:5000; Abcam), and enhanced chemiluminescence (Pierce, Rockford, IL, USA) was used to measure the blots.

Statistical analyses

All the results, obtained from triplicate parallel experiments, were expressed as Mean±SD. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis. The comparison of significant difference was determined by Student’s t test and one-way Analysis of Variance. A p-value <0.05 was considered as statistically significant difference.
Results

Asiatic acid attenuated high fat diet-induced dyslipidemia

To establish the osteoporotic animal model, mice were fed with high fat diet for 16 weeks. Data from biochemical analyzer showed that HFD induced elevated levels of TG, TC and LDL (Figure 1), but reduced HDL in serum samples of mice (Figure 1). However, administration of asiatic acid reduced levels of TG, TC and LDL, and enhanced HDL to ameliorate dyslipidemia (Figure 1), suggesting that asiatic acid attenuated high fat diet-induced dyslipidemia during development of osteoporosis.

Asiatic acid attenuated high fat diet-induced oxidative stress

The effect of asiatic acid on oxidative stress was also investigated. The production of MDA and ROS was up-regulated, while the expression of SOD and GSH-Px was down-regulated in mice post HFD, which were restored by asiatic acid administration (Figure 2), suggesting the antioxidative role of asiatic acid in osteoporosis.

Asiatic acid promoted osteogenic differentiation in HFD-induced osteoporosis mice model

Levels of osteoporotic markers, including ALP and P1NP, were decreased in mice fed with HFD (Figure 3), while CTX-1 was increased in osteoporotic mice (Figure 3). Asiatic acid attenuated HFD-induced increase of CTX-1 and decrease of ALP and P1NP (Figure 3). Moreover, H&E staining showed that HFD reduced trabecular number (Figure 4), while asiatic acid administration enhanced the
trabecular number (Figure 4). Additionally, HFD-fed rats had lower BFR/BS and BV/TV than that in control group (Figure 4B). However, asiatic acid administration increased levels of BFR/BS and BV/TV (Figure 4B). Asiatic acid also attenuated the HFD-induced decrease of MAR in HFD-fed rats (Figure 4C) to induce osteogenic differentiation in osteoporotic mice. These results indicated that asiatic acid promoted osteogenic differentiation in osteoporotic mice.

Asiatic acid promoted osteogenic differentiation of bone marrow cells

Bone marrow cells were isolated from long bones of mice to determine the effect of asiatic acid on the osteogenic potential. The ALP activity of bone marrow cells was reduced in HFD-fed mice (Figure 5A), which was restored by asiatic acid administration (Figure 5A). The mineralizing capability of bone marrow cells was also reduced in osteoporotic mice (Figure 5B). Asiatic acid enhanced Alizarin-Red-stained calcium nodule numbers to promote osteogenic differentiation (Figure 5B). Moreover, asiatic acid attenuated the HFD-induced decrease of osteoblast-specific markers, including collagen type-1, RUNX2 and OCN, in bone marrow cells (Figure 5C), revealing the promotive effect of asiatic acid on osteogenic differentiation of bone marrow cells. Furthermore, the adipogenic differentiation capacity of bone marrow cells was elevated in HFD-fed rats, which was suppressed by asiatic acid administration (Figure 5D).
Asiatic acid promoted activation of SIRT1/FOXO1 to attenuate osteoporosis

To elucidate the mechanism involved in asiatic acid-mediated osteoporosis, proteins in the SIRT1/FOXO1 pathway were analyzed by western blot. SIRT1 was decreased in bone marrow cells isolated from HFD-fed mice (Figure 6), but increased by asiatic acid administration (Figure 6). Up-regulation of SIRT1 by asiatic acid reduced the level of Ac-FOXO1 and enhanced Nu-FOXO1 through deacetylation (Figure 6). These results suggested that asiatic acid might regulate osteoporosis-associated oxidative stress through activation of the SIRT1/FOXO1 signaling pathway.

Discussion

Previous studies have shown that asiatic acid attenuated bone loss, inhibited osteoclastogenesis and promoted osteogenic differentiation in ovariectomized-induced osteoporotic mice (Tomlinson et al., 2019). Moreover, asiatic acid possessed antioxidative activity to protect against fulminant hepatic failure (Lv et al., 2017). HFD-induced damaged spermatogenesis (Miao et al., 2018), hepatic lipid accumulation and insulin resistance (Yan et al., 2014) were alleviated by asiatic acid. The effects of asiatic acid on oxidative stress and osteogenic differentiation of HFD-induced osteoporotic mice were investigated in this study.

Disorder of lipid metabolism has been shown to be associated with osteoporosis and is a detrimental factor to various organs (Tian and Yu, 2015). Serum levels of HDL and TC functioned as indicators of postmenopausal osteoporosis patients (Sivas et al., 2009). Asiatic acid has been reported to possess antidiabetic potential through
attenuation of metabolic dysregulation of lipid profile in streptozotocin-nicotinamide-induced diabetic rats (Swapna et al., 2019). Our results showed that asiatic acid increased HDL, decreased TG, TC and LDL in HFD-induced osteogenic mice, suggesting asiatic acid had antidyslipidemic effect against osteoporosis. (this section is unclear and requires revision)

Accumulating evidence has shown that oxidative stress participates in the pathogenesis of osteoporosis through regulation of osteoblasts and osteoclasts differentiation (Abdollahi et al., 2005). Accumulation of reactive oxygen species, as well as the increase of end product of lipid peroxidation, MDA, are enhanced in bone disorders (Abdollahi et al., 2005). Moreover, the activities of antioxidative enzymes, including SOD and GSH-Px, are reduced during osteoporosis (Abdollahi et al., 2005). Our results demonstrated that asiatic acid reduced levels of MDA and ROS, enhanced SOD and GSH-Px in osteoporotic mice, (this section is unclear and requires revision) suggesting its antioxidative effect against osteoporosis. Enhanced oxidative stress repressed osteogenic differentiation with reduced osteogenic differentiation marker, ALP, through regulating autophagy (Yang et al., 2021) or hedgehog (Kim et al., 2010) signaling pathways. Asiatic acid has been shown to retard osteoclastic bone resorption and attenuate bone loss in NF-κB/TGF-β (Huang et al., 2019) or NFATc1 (Tomlinson et al., 2019) dependent pathways. This study indicated that asiatic acid promoted osteogenic differentiation in HFD-induced osteoporosis mice through decrease of CTX-1 (bone resorption marker), increase of ALP and P1NP (bone loss predictor). (this section is unclear and requires revision)Moreover, osteoblast-specific markers
(collagen type-1, RUNX2 and OCN) in bone marrow cells were also up-regulated by asiatic acid. These results revealed that asiatic acid suppressed osteoporosis-associated oxidative stress and promoted osteogenic differentiation to attenuate osteoporosis.

SIRT1 functions as a NAD\(^+\)-dependent deacetylase to regulate pathways involved in obesity and ageing-related metabolic disorders (Pardo and Borick, 2020). Ageing mice with SIRT1 overexpression showed lower incidence of osteoporosis (Tomlinson et al., 2019). Administration of activator of SIRT1, SRT2104, enhanced bone mineral density and suppressed bone resorption to attenuate age-related osteoporosis (Mercken et al., 2014). Suppression of SIRT1 reversed the suppressive effects of asiatic acid on neuroinflammation in BV2 microglia (Qian et al., 2018). (this section is unclear and requires revision) Our results showed that protein expression of SIRT1 was increased in osteoporotic mice post asiatic acid administration. In addition, hydrogen peroxide-induced apoptosis of osteoblasts was suppressed by SIRT1 in a FOXO1/β-catenin dependent manner (Yao et al., 2018). FOXO1, a transcriptional factor and downstream target of SIRT1, is involved in cell proliferation and oxidative stress of osteoblasts (Eelen et al., 2013). FOXO1 was also implicated in the 1α,25-Dihydroxyvitamin D\(_3\)-mediated oxidative stress of osteogenesis (Xiong et al., 2017). Activation of SIRT1 by resveratrol reduced acetylation of FOXO1 and enhanced nuclear translocation of FOXO1 to regulate redox balance and promote osteogenic differentiation during development of osteoporosis (Jiang et al., 2020). Protein expression of Ac-FOXO1 was reduced, while
Nu-FOXO1 was enhanced in osteoporotic mice post asiatic acid administration. These results suggested that asiatic acid might promote osteogenic differentiation in osteoporosis through reducing oxidative stress in a SIRT1/FOXO1 axis dependent pathway.

In conclusion, asiatic acid attenuated HFD-induced dyslipidemia through increasing HDL, and decreasing TG, TC and LDL. Moreover, HFD-induced oxidative stress was also suppressed by asiatic acid with reduced MDA and ROS, enhanced SOD and GSH-Px. Asiatic acid also restored osteoblast functions in HFD-induced osteoporosis mice. Activation of the SIRT1/FOXO1 pathway might be involved in the antioxidative effect of asiatic acid on HFD-induced osteoporosis, providing a novel strategy for the prevention of osteoporosis.

Acknowledgements

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Funding

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Competing interests

The authors state that there are no conflicts of interest to disclose.
Ethics approval

Ethical approval was obtained from the Ethics Committee of Affiliated Hospital of Guangdong Medical University.

Contribution of authors

Xiaosi Chen and Dengpeng Han designed the study, supervised the data collection, Tianfeng Liu and Chengshuo Huang analyzed the data, interpreted the data, Zibing Hu, Xiaoyan Tan and Shaoke Wu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Figure legends

Figure 1. Asiatic acid attenuated high fat diet-induced dyslipidemia

Asiatic acid attenuated high fat diet-induced decrease of HDL, and increase of TG, TC and LDL in serum samples of mice. *, **, p < 0.05, p < 0.01. N = 3. high-density lipoprotein (HDL), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), high-fat-diet (HFD).

Figure 2. Asiatic acid attenuated high fat diet-induced oxidative stress

Asiatic acid attenuated high fat diet-induced decrease of SOD and GSH-Px, and increase of MDA and ROS in serum samples of mice. (this section is unclear and requires revision) *, **, p < 0.05, p < 0.01. N = 3. malondialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px).
Figure 3. Asiatic acid promoted osteogenic differentiation in HFD-induced osteoporosis mice

Asiatic acid attenuated high fat diet-induced increase of CTX-1, and decrease of ALP and P1NP in serum samples of mice. (this section is unclear and requires revision) *, **, $p < 0.05$, $p < 0.01$. N = 3. c-telopeptide of type 1 collagen (CTX-1), alkaline phosphatase (ALP), procollagen type 1 N-terminal propeptide (P1NP).

Figure 4. Asiatic acid attenuated deterioration of bone microstructure in HFD-induced osteoporosis mice

(A) H&E staining of distal femurs showed that HFD induced decrease of trabecular number, while asiatic acid administration enhanced the trabecular number. Scale bars: 100 µm.

(B) HFD-induced BFR/BS and BV/TV in rats were increased by asiatic acid administration.

(C) Representative images and quantitative analysis of calcein double labelling showed that HFD-induced MAR in rats was increased by asiatic acid administration. **, $p < 0.01$. N = 3. mineral apposition rate (MAR), bone formation rate/trabecular bone surface (BFR/BS) and trabecular bone volume/total tissue area (BV/TV).
Figure 5. Asiatic acid promoted osteogenic differentiation of bone marrow cells

(A) Asiatic acid attenuated high fat diet-induced decrease of ALP activity in bone marrow cells.

(B) Alizarin-Red-stained of bone marrow cells showed that asiatic acid enhanced calcium nodule numbers to promote the osteogenic differentiation. Scale bars: 100 µm.

(C) Asiatic acid attenuated HFD-induced decrease of collagen type-1, RUNX2 and OCN in bone marrow cells.

(D) Asiatic acid attenuated HFD-induced adipogenic differentiation capacity of bone marrow cells. Scale bars: 100 µm. *, **, p < 0.05, p < 0.01. N = 3. collagen type-1 (Col-1), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN).

Figure 6. Asiatic acid promoted activation of SIRT1/FOXO1 to attenuate osteoporosis

Asiatic acid attenuated HFD-induced increase of Ac-FOXO1, decrease of SIRT1 and Nu-FOXO1 in bone marrow cells. (this section is unclear and requires revision) *, **, p < 0.05, p < 0.01. N = 3. nuclear FOXO1 (Nu-FOXO1), nuclear FOXO1 (Nu-FOXO1)
The images depict bar charts showing the effects of different treatments on various parameters.

**TG (mmol/L)**
- HFD -
- HFD +
- AA (mg/kg) - 25
- AA (mg/kg) - 50

**TC (mmol/L)**
- HFD -
- HFD +
- AA (mg/kg) - 25
- AA (mg/kg) - 50

**LDL (mmol/L)**
- HFD -
- HFD +
- AA (mg/kg) - 25
- AA (mg/kg) - 50

**HDL (mmol/L)**
- HFD -
- HFD +
- AA (mg/kg) - 25
- AA (mg/kg) - 50
A

HFD+AA (25mg/kg)

HFD+AA (50mg/kg)

B

MAR (µm/d)

HFD
AA (mg/kg)
- + 25 50

BFR/BS (µm²/µm²/day)

HFD
AA (mg/kg)
- + 25 50

BV/TV (%)

HFD
AA (mg/kg)
- + 25 50

C

Control

HFD

HFD+AA (25mg/kg)

HFD+AA (50mg/kg)
**HISTOLOGY AND HISTOPATHOLOGY**

**A**

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**B**

- Control
- HFD
- HFD+AA (25mg/kg)
- HFD+AA (50mg/kg)

**C**

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**D**

- Control
- HFD
- HFD+AA (25mg/kg)
- HFD+AA (50mg/kg)
**SIRT1**

- **Ac-FOXO1**
- **GAPDH**
- **Nu-FOXO1**
- **Histone H3**

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**Relative SIRT1 expression**

- HFD
- AA (mg/kg) - - + + + +

**Relative Ac-FOXO1 expression**

- HFD
- AA (mg/kg) - - 25 50

**Relative Nu-FOXO1 expression**

- HFD
- AA (mg/kg) - + + +