Synergistic Effect of Sesamin and \(\gamma\)-Tocotrienol on Promoting Osteoblast Differentiation via AMPK Signaling

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Abstract

**Background:** Sesamin is a rich phytochemical found in sesame seed oil that can promote osteoblast differentiation of rat BMSCs and improve rat bone structure by regulating Wnt/\(\beta\)-Catenin pathway. Combined sesamin and \(\gamma\)-Tocotrienol (\(\gamma\)-T3) have been clarified to inhibit the proliferation of breast cancer cells, but their role in osteoporosis has not been explored. This paper aimed to discuss the synergistic effect of sesamin and \(\gamma\)-T3 in osteoporosis and disclose the underlying mechanism.

**Materials and methods:** CCK-8 assay was to appraise the proliferation of hBMSCs after treated with sesamin and \(\gamma\)-T3. Moreover, the proteins in AMPK signaling in osteoblasts pretreated with AMPK inhibitor compound C (CC) were detected after the induction of sesamin and \(\gamma\)-T3. Then, CCK-8, ALP assay and ARS staining were used to analyze whether the proliferation and osteoblast differentiation of hBMSCs was via AMPK pathway. RT-qPCR and western blot were conducted to quantify the levels of markers in osteoblasts.

**Results:** It was determined that 5 g/mL sesamin and 1 \(\mu\)M \(\gamma\)-T3 exerted obvious influences on the viability of hBMSCs. Moreover, the co-treatment of sesamin and \(\gamma\)-T3 elevated the protein levels of related factors in AMPK pathway, which was reversed by CC. Furthermore, The proliferation and osteoblast differentiation exhibited remarkable increments upon exposure to both sesamin and \(\gamma\)-T3, whereas CC abolished these effects.

**Conclusion:** In conclusion, the present study presented the first line of evidence to verify the synergistic effects of sesamin and \(\gamma\)-T3 on alleviating osteoporosis, and revealed their effects were realized by modulating the AMPK pathway. This paper has indicated the great potential of combined sesamin and \(\gamma\)-T3 in osteoporosis treatment.

**Keywords**
sesamin, \(\gamma\)-tocotrienol, osteoblast differentiation, AMPK

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**Introduction**

Osteoporosis is a systemic disorder that contributes to decreased bone mass and increased susceptibility to bone fracture\(^1\). As a consequence of imbalance in the bone remodeling process, the bone formation ability of patients will be weakened, leading to bone fragility and even bone fracture\(^2\). The current therapeutic drugs for osteoporosis including bisphosphonate, osteocalcin and estrogen which aim at enhancing bone synthesis can only achieve few improvements\(^3\). Thus, identifying novel targets or drugs is of great necessity for the management of osteoporosis and improvement of patients’ health.

Sesamin (Figure 1A) is a rich phytochemical found in sesame seed oil. The recent literature has supported that sesamin can promote osteoblast differentiation of rat BMSCs and improve rat bone structure by regulating Wnt/\(\beta\)-Catenin pathway\(^4\). Besides the protection role in femoral head from necrosis by inhibiting ROS-induced osteoblast apoptosis, it can directly affect the functions of osteoblasts by stimulating the expression of essential genes and key enzymes in the process of bone mineralization possibly via the activation of p38 and ERK1/2 MAPK signaling pathway\(^5\). Thus, the promotive effect of sesamin on osteoblast differentiation has been confirmed.
It is reported that sesamin can improve the bioavailability of tocopherol and tocotrienol in vivo. γ-Tocotrienol (γ-T3; Figure 1B) is a natural form of vitamin E, with which sesamin can exert anti-proliferative effects on breast cancer cells. In animal models, γ-T3 can prevent bone loss caused by oophorectomy through HMG-CoA reductase. Furthermore, the therapeutic potential of γ-T3 in repairing bone injury caused by long-term smoking has been found. A moderate concentration of γ-T3 has antioxidant activity as it protects osteoblasts from oxidative stress. However, the impacts of γ-T3 on osteoblast differentiation has not been illustrated. It is speculated that sesamin combined with γ-T3 can synergistically promote osteoblast differentiation.

AMPK was found to be close association with γ-T3 and sesamin. It is well documented that γ-T3 activates AMPK and autophagy signals to synergistically inhibit adipogenic transformation of hASCs into adipocytes. The application of sesamin to liver HepaRG and intestinal LS174T cells reduces LXR activation, inhibits the expression of downstream target genes, and significantly improves the accumulation of lipids in HepaRG cells, possibly due to its relation to activation of AMP activated protein kinase (AMPK) signaling pathway. Moreover, AMPK signaling has been recently identified as a crucial participant in the osteoblast differentiation, as suggested by the evidence that chrysophanol promotes osteoblast differentiation through AMPK/Smad1/5/9 expression in vitro and in vivo. Thus, we postulated that AMPK might mediate involved in the effects of γ-T3 and sesamin on osteoporosis.

In the present study, we will innovatively discuss the synergistic effects of sesamin and γ-T3 on osteoporosis by detecting their combined roles in the proliferation and mineralization of osteoblasts and elaborate the latent regulatory mechanism.

Materials and Methods

Cell Culture and Treatment

The procedure of cell culture was conducted as previously reported. Human bone marrow mesenchymal stem cells (hBMSCs), purchased from Nanjing Saigen Biotechnology Co., Ltd, were cultured in α-Minimum essential medium containing 10% fetal bovine serum, 100 mg/L streptomycin and 100 U/L penicillin α-Minimum essential medium in a humid environment at 37 °C and 5% CO2. For osteogenic differentiation, when cells reached 80 to 90% confluence, 10 mM β-Glycerophosphate, 100 nM dexamethasone and 200 μM ascorbic acid (osteogenic inducer) were added to the medium and incubated for 14 days, and osteogenic induction medium (OM) was changed every 3 days. Control cells were cultured in the medium without osteogenic inducer.

Sesamin (S9314;Sigma-Aldrich LLC., USA) was dissolved in DMSO. γ-T3 was procured from Sigma Chemical Co (St. Louis, MO, USA). Cells were exposed to 1, 2.5, 5, 10 mg/mL doses of sesamin and 1, 5, 10 mM doses of γ-T3 AMPK inhibitor compound C (CC), which was bought from Calbiochem (Nottingham, UK), was used to pretreat the cells for 2 h.

Cell Counting Kit-8 (CCK-8)

Cell viability was detected using CCK-8 (BeyoTime Institute of Biotechnology). Cells were first plated in 96-well plates at a density of 5×10^4 cells/well. Subsequently, 10 µl CCK-8 solution was added and incubated at 37 °C for 4 h. The optical density of each well was determined at 450 nm by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western Blot

Total proteins were extracted from the cells in lysis buffer (Cell Signaling Technologies, USA). Quantification of protein concentration was conducted by a bicinchoninic acid (BCA) assay kit (Beyotime, China). Equal amounts of protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in 0.1 M Tris-buffered saline-0.1% Tween-20 buffer (TBST) for 2 h. Then, the membranes were incubated with the primary antibodies and horseradish peroxidase-conjugated secondary antibody for 1 h. Blots were developed with an enhanced chemiluminescence reagent (Amersham Biosciences, USA) and quantified by densitometric scanning and analyses using an ImageQuant LAS 4000 system (Fujifilm, Tokyo, Japan).

Detection of Alkaline Phosphatase (ALP) Activity

The ALP activity was measured at 14 days following cell culture. Cells were incubated in 96-well plates and washed by PBS for three times, followed by the addition and sonication of distilled water to each well. Total protein was then extracted and quantified by BCA protein assay kit (Pierce, Rockford, IL). Alkaline phosphatase (ALP) activity was assayed by a fluorescence detection kit per the manufacturer’s protocol. A standard curve was prepared by p-nitrophenol. Each value was normalized to the protein concentration.

Alizarin Red S (ARS) Staining

14 days after treatment, cell were fixed with 70% ice-cold ethanol for 1 h. After washed three times with PBS, cells were incubated with 20 mM of alizarin red solution for 15 min at room temperature (RT). The stained cells were photographed after washed with PBS three times. Then, 10% cetylpyridinium chloride was added to extract the stained cells. Absorbance was recorded at 562 nm using a spectrophotometer (NanoDrop Technologies, USA) and analyzed using Image J software (National Institutes of Health).
Figure 1. The effects of sesamin and γ-T3 on the cell viability of hBMSCs. The chemical structure of (A) sesamin and (B) γ-T3. CCK-8 assay determined the effects of (C) sesamin and (D) γ-T3 on the cell viability of hBMSCs. *p < 0.05, ***p < 0.001.

Figure 2. Sesamin and γ-T3 activates the AMPK signaling. Western blot analyzed the protein levels of p-AMPK and p-Samd1 in CC-pretreated hBMSCs induced by sesamin and γ-T3. *p < 0.05, ***p < 0.001 vs control. $p < 0.001$ vs OM group. $++p < 0.001$ vs OM + sesamin group. $+++p < 0.001$ vs OM + γ-T3 group. $++++p < 0.001$ vs OM + sesamin + γ-T3 group.
To demonstrate that co-treatment with sesamin and γ-T3 could exert better stimulative effect on osteoblast differentiation as a consequence of joint effects of sesamin and γ-T3, pretreatment with CC dramatically reduced the phosphorylation of AMPK and Smad-1, while co-treatment with sesamin and γ-T3 could trigger more increments in their protein levels, as compared to OM group (Figure 2). Further results that pretreatment with 1 μmol/L CC, the AMPK inhibitor, dramatically reduced the phosphorylation of AMPK and Smad-1 indicated that co-treatment with sesamin and γ-T3 activated the AMPK signaling. In summary, sesamin and γ-T3 served as a stimulator of AMPK signaling.

The Combined Effect of Sesamin and γ-T3 on the Osteoblast Differentiation of hBMSCs via AMPK Signaling

Osteoblast differentiation is conducive to the bone remodeling and treatment of osteoporosis, and thus we conducted ALP assay to validate the combine effects of sesamin and γ-T3 on osteoblast differentiation. It turned out that sesamin and γ-T3 respectively elevated the ALP enzyme activity, albeit not to the extent where co-treatment with sesamin and γ-T3 did (Figure 4A-B). Intriguingly, the enhanced osteoblast differentiation as a consequence of joint effects of sesamin and γ-T3 was crippled by CC pretreatment. Concomitantly, co-treatment of sesamin and γ-T3 displayed more obvious mineral deposition than treatment of
sesamin or γ-T3 alone, whereas CC pretreatment abated the potential of hBMSCs to form mineralized nodules (Figure 4C-D). hBMSCs were cultured in OM for 14 days, the marker so for osteoblasts, OPN, OCN, Runx2, and Osterix, exhibited similar trends to the osteoblast differentiation and mineralization that had been displayed (Figure 5A-B). Taken together, sesamin and γ-T3 mediated the AMPK signaling to contribute to hBMSCs osteoblast differentiation.

Discussion

Osteoporosis is presented as a result of high bone turnover with abnormal bone mass, and recent epidemiologic statistics has estimated that implying the possible occurrence of half of osteoporotic fractures in Asia by 2050 has prompted our research in recognizing novel targets or drugs for the treatment of osteoporosis14,15. Skeletal growth, repair and remodeling, which are known as critical processes for bone formation, are implicated in the synthesis and deposition of mineralizing extracellular matrix by osteoblasts16.

It was previously delineated by an in vitro and in vivo experimental study that sesamin enhanced osteoblast differentiation by Wnt/β-catenin signaling, thereby preventing osteoporosis4. The following study that the potential of innovatively combined sesamin and γ-T3 for the treatment of breast cancer rats has inspired us to validate whether combined sesamin and γ-T3 are capable of improving the therapeutic effects in current osteoporosis6. Interestingly, the favorable effects of γ-T3 on bone have been noticed to be attributed to its inhibition of osteoclast formation and protection against bone loss17. In the present study, as observed by CCK-8 analysis, respective administrations of sesamin and γ-T3 both induced higher proliferation of hBMSCs on the 7th and 14th day, while co-treatment with sesamin and γ-T3 achieved higher efficiency in promoting the cell proliferation. This result preliminarily determined the combinational use of sesamin and γ-T3 as an ideal choice for the management of osteoporosis.

AMPK pathway is also of substantial value in bone physiology owing to its enhancement in bone formation and bone mass18. Ghrelin, a potent appetite stimulator which also affects bone mass, enhances the AMPK phosphorylation and activity in ROS17/2.8 cells, suggesting the implication of AMPK activity in regulating osteoblast functions by ghrelin19,20. Importantly, AMPK agonist stimulated the proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells21. As expected, treatment of sesamin or γ-T3 alone promoted the phosphorylation of AMPK and Smad1, whereas this effect was enhanced by their synergistic effects. Herein, to further substantiate whether sesamin and γ-T3 exerted potent activities on promoting the proliferation of osteoblasts via AMPK pathway, we pretreated the cells with AMPK inhibitor CC, identifying the significant involvement of AMPK in stimulating the effects of combined sesamin and γ-T3 on osteoblasts. Consistently, CC was previously

Figure 4. The combined effect of sesamin and γ-T3 on the osteoblast differentiation of hBMSCs via AMPK signaling. (A-B) The ALP activity and (C-D) mineralization of hBMSCs in sesamin and γ-T3-induced osteoblasts upon CC pretreatment. **p < 0.01, ***p < 0.001 vs control. $p < 0.05$ vs OM group. **p < 0.01, ***p < 0.001 vs OM + sesamin group. *p < 0.05, ++p < 0.01 vs OM + γ-T3 group. #p < 0.05, ##p < 0.01 vs OM + γ-T3 group.
uncovered to decrease AMPKα phosphorylation in primary osteoblasts and ROS17/2.8 cells.20

The importance of AMPK in regulating osteoblast differentiation has been highlighted by existing studies. For instance, both microRNA-9 and 3,5-dicaffeoyl-epi-quinic acid enhanced osteoblast differentiation by activating AMPK activity.22 Furthermore, accumulating evidence has suggested that AMPK regulators modulate bone cell differentiation and function, demonstrating the interplay between AMPK and osteoblast differentiation.21,23 Since the necessity of sesamin in inducing osteoblast differentiation has been clarified, we next made an attempt to elucidate the combined effects of sesamin and γ-T3 on stimulating osteoblast differentiation via AMPK pathway. Our experimental results showed that the nodules formation of osteoblasts was enhanced due to the combinational use of sesamin and γ-T3, whereas CC partially reversed this effect. ALP, OPN, OCN, Runx2, and Osterix are recognized as possible biomarkers in the progression of osteoporosis.4,24–26 Patients with this disease exhibited lower levels of ALP, OCN, Runx2, and Osterix in comparison to healthy controls. The increased levels of these biomarkers due to the combined treatment of sesamin and γ-T3 indicated the protective role of combined sesamin and γ-T3 in osteoporosis, and the cancellation of this effect by CC pretreatment indirectly demonstrated the involvement of AMPK pathway in sesamin and γ-T3-mediated osteoporosis.

**Conclusion**

In conclusion, the present study presented the first line of evidence elucidating the synergistic effects of sesamin and γ-T3 on alleviating osteoporosis, and revealed their effects were realized by modulating the AMPK pathway. This paper has indicated the great potential of the combinational use of sesamin and γ-T3 in osteoporosis treatment.
Declaration of Conflicting Interests
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Ethical Approval
Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent
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Trial Registration
Not applicable, because this article does not contain any clinical trials.
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