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# Naringin protects human adipose-derived mesenchymal stem cells against hydrogen peroxide-induced inhibition of osteogenic differentiation

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ABSTRACT

Extensive evidence indicates that oxidative stress plays a pivotal role in the development of osteoporosis. We show that naringin, a natural antioxidant and anti-inflammatory compound, effectively protects human adipose-derived mesenchymal stem cells (hADMSCs) against hydrogen peroxide ($H_2O_2$)-induced inhibition of osteogenic differentiation. Naringin increased viability of hAMDSCs and attenuated $H_2O_2$-induced cytotoxicity. Naringin also reversed $H_2O_2$-induced oxidative stress. Oxidative stress induced by $H_2O_2$ inhibits osteogenic differentiation by decreasing alkaline phosphatase (ALP) activity, calcium content and mRNA expression levels of osteogenesis marker genes *RUNX2* and *OSX* in hADMSCs. However, addition of naringin leads to a significant recovery, suggesting the protective effects of naringin against $H_2O_2$-induced inhibition of osteogenic differentiation. Furthermore, the $H_2O_2$-induced decrease of protein expressions of β-catenin and clyclin D1, two important transcriptional regulators of Wnt-signaling, was successfully rescued by naringin treatment. Also, in the presence of Wnt inhibitor DKK-1, naringin is no longer effective in stimulating ALP activity, increasing calcium content and mRNA expression levels of *RUNX2* and *OSX* in $H_2O_2$-exposed hADMSCs. These data clearly demonstrates that naringin protects hADMSCs against oxidative stress-induced inhibition of osteogenic differentiation, which may involve Wnt signaling pathway. Our work suggests that naringin may be a useful addition to the treatment armamentarium for osteoporosis and activation of Wnt signaling may represent attractive therapeutic strategy for the treatment of degenerative disease of bone tissue.



## 1. Introduction

Osteoporosis is characterized by low bone mineral density and microarchitecture deterioration and leads to degenerative disease of bone tissue. Apart from estrogen withdrawal which happens more commonly in the senior society, extensive studies in animal experimental models and epidemiological evidence in humans suggest that oxidative stress is a major contributor to the development of osteoporosis [1]. Oxidative stress is a result of excessive generation of intracellular reactive oxygen species (ROS) which cause cell damage by oxidation and nitration of DNA/RNA, proteins and lipids [2]. ROS interacts with various transcriptional pathways that are essential for mesenchymal stem cell (MSC) differentiation including the Wnt and FOXO signaling cascades [3]. Oxidative stress induced by hydrogen peroxide ($H_2O_2$), xanthine or lipid oxidation products has also been reported to contribute to osteoporosis by inhibiting osteoblastic differentiation of MSC and bone formation [2].

Human adipose-derived mesenchymal stem cells (hADMSCs) provide a promising future for regenerative medicine because there are multipotent and can differentiate into various cell types, including osteocytes [4]. The surface immunophenotype of ADMSCs resembles MSCs and express adult stem cell markers such as CD29 and CD105 but not CD34 and CD45 [5]. Osteogenesis by hADMSCs successfully occurs under 3D culture on a wide variety of scaffolds [6] and bone generation is also enhanced by using hADMSCs on demineralized bone allografts [7]. Compared to bone marrow-derived MSCs (golden standard adult MSCs), hADMSCs are easily accessible, have higher yield of stem cells and more potent for lineage-specific differentiation [4]. Hence, hADMSCs are

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promising candidate for the treatment of bone disease.

Naringin is the main active component of *rhizoma drynariae* (a traditional Chinese herbal medicine) used to treat orthopaedic disorders and bone healing [8]. Naringin enhances rat osteoblasts proliferation, differentiation and maturation via stimulating the secretion of osteocalcin, osteopontin, bone morphogenetic protein-2 and collagen I [9]. Naringin is found to effectively reverses ovariectomy-associated osteoporosis *in vivo* [10]. Recently, accumulating evidence indicate that naringin has protective effects on $H_2O_2$ or ischemia-reperfusion induced oxidative stress in various type of cells [11,12], suggesting that naringin have antioxidant properties.

Considering the importance of oxidative stress in the pathogenesis of osteoporosis, we hypothesized that naringin might attenuate the deleterious effects of oxidative stress on osteoblastic differentiation. In this work, we investigated the effects of naringin on $H_2O_2$-induced inhibition of osteoblastic differentiation of hADMSCs and the underlying mechanisms. We found that naringin increased the viability of hADMSCs and effectively reversed the deleterious effects of oxidative stress on osteogenic differentiation. Furthermore, we identified Wnt signaling pathway as the possible molecular target of the protective effects of naringin on oxidative stress induced inhibition of osteogenic differentiation.

## 2. Materials and methods

### 2.1. Culture of human adipose-derived mesenchymal stem cells (hADMSCs) and induction of osteoblast differentiation

hADMSCs were isolated from adipose tissue as previously described [13] and then cultured in serum- and xeno-free MesenCult-XF medium (Stemcell technologies, US). Osteoblast differentiation was induced by culturing hADMSCs in the differentiation medium containing α-minimum essential medium (α-MEM) (Life Technologies, US), 20% fetal bovine serum (FBS) (Hyclone, UK) and 2 mM L-glutamine (Life Technologies). Media was replaced with fresh ones every 2–3 days. The subject gave informed consent, and patient anonymity has been preserved. This study was approved by the ethics committee of Affiliated Hospital of Jining Medical University.

### 2.2. hADMSCs treatment with naringin, $H_2O_2$ or the combination of both

Naringin, $H_2O_2$ (Sigma–Aldrich, US) or the combination of both was added to the differentiation medium for the treatment. Cells cultured in medium without naringin or $H_2O_2$ were used as control. As a positive control, N-acetyl-L-cysteine (NAC, 1 mM, Sigma) was used during the culture. After 1–2 days culture, cells were tested for viability using MTT assay. After 8 days culture, cells were tested for early stage of osteogenic differentiation using alkaline phosphatase (ALP) staining and activity assay. After 16 days culture, cells were tested for late stage of osteogenic differentiation using alizarin red S staining and calcium assay. For the rescue experiment, Wnt inhibitor DKK-1 was added to the differentiation medium together with naringin and $H_2O_2$.

### 2.3. Immunofluorescence labeling and flow cytometry

Isolated hADMSCs were stained with fluorochrome-conjugated monoclonal antibodies against mesenchymal stem-cell surface marker proteins for 30 min in a dark room. The antibodies are anti-CD105-FITC (Serotec, UK), anti-CD29-FITC, anti-CD45-FITC, anti-CD34-FITC and mouse fluorochrome-conjugated isotype control IgG antibody (Dinona) as negative control. Stained cells were washed with PBS for three times prior to flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, CA).

### 2.4. Measurement of oxidative stress

Oxidative stress was determined by measuring the activity of superoxide dismutase (SOD), the level of glutathione (GSH) and malondialdehyde (MDA). SOD activity was measured by Superoxide Dismutase Assay Kit (Cayman Chemical, UK) according to the manufacturer's instructions. Briefly, hADMSCs lysate was added to the reaction mixture. Enzyme reaction was then initiated by adding NADH and stopped by adding glacial acetic acid. The absorbance at 560 nm was measured using a SpectraMAX 250 microplate reader (Molecular De vices, US). GSH levels were measured using the same method described previously [14]. Briefly, hADMSCs lysate was heated with thiobarbituric acid for 40 min at 95 °C and then centrifuged at 3500 rpm for 10 min. The supernatant was collected and the absorbance at 532 nm was measured. MDA levels were measured using the same method described previously [15]. Briefly, hADMSCs lysate was mixed with 5, 5-dithiobes-(2-ni-trobenzoic acid) (DTNB) and phosphate buffer and then absorbance at 412 nm was measured.

### 2.5. Alkaline phosphatase (ALP) staining and activity measurement

Osteogenesis of hADMSCs was induced in differentiation medium for 8 days. For ALP staining, cells were rinsed with PBS, fixed with 4% paraformaldehyde (Sigma–Aldrich) and stained with 0.1 mg/ml of naphthol AS-MX phosphate, 0.5% N, N-dimethylformadie, fast blue BB salt, and 2 mM $MgCl_2$ in Tris–HCl buffer for 30 min. Cells were washed with PBS for three times prior to cell imaging using a light microscope Olympus IX71 (Olympus Corporation, Japan). For ALP activity measurement, cells were lysed and then spin down at 12,000 × g for 10 min. The protein levels in the supernatant were determined by bicinchoninic acid protein assay kit (Thermo Scientific) and used to normalize ALP activity. 50 μl of lysed sample was incubated with 50 μl p-nitrophenyl phosphate (pNPP; Sigma–Aldrich) substrate solution for 15 min at 37 °C. The reaction was stopped by adding 25 μl NaOH. Absorbance was measured at 405 nm. The relative ALP activity was first normalized according to the total protein content of cell lysate and then calculated relative to their treatment controls.

### 2.6. Alizarin red S (ARS) staining and calcium assay

Alizarin red staining was performed after 16 days of culturing hADMSCs in the differentiation medium. Briefly, cells were rinsed with PBS, fixed with 4% paraformaldehyde and stained with 1% alizarin red S solution (Sigma–Aldrich) for 10 min. Orange red staining indicated location and intensity of the calcium deposition. The presence of calcium was observed using a light microscope Olympus IX71 (Olympus Corporation). The calcium deposition was measured using Calcium Assay kit (Genzyme Dianostics, Canada) according to manufacturer's instructions. Briefly, cells were incubated with 1 M acetic acide at overnight 4 °C. Cell extract at 15 μl was mixed with 150 μl Calcium Assay reagent and the absorbance at 650 nm was measured using SpectraMAX 250 microplate reader.

### 2.7. Cell viability assay

Cell viability were investigated using MTT (reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product) assay kit (Sigma–Aldrich) according to manufacturer's instructions. Time course analysis of cell viability was also examined at 0, 12, 24, 36 and 48 h post-treatment.