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Protective effects of myricitrin against osteoporosis via reducing reactive oxygen species and bone-resorbing cytokines

Qiang Huang [1], Bo Gao [1], Long Wang, Ya-Qian Hu, Wei-Guang Lu, Liu Yang *, Zhuo-Jing Luo *, Jian Liu **

Institute of Orthopedic Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, People's Republic of China

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ABSTRACT

Oxidative stress is a crucial pathogenic factor in the development of osteoporosis. Myricitrin, isolated from *Myrica cerifera*, is a potent antioxidant. We hypothesized that myricitrin possessed protective effects against osteoporosis by partially reducing reactive oxygen species (ROS) and bone-resorbing cytokines in osteoblastic MC3T3-E1 cells and human bone marrow stromal cells (hBMSCs). We investigated myricitrin on osteogenic differentiation under oxidative stress. Hydrogen peroxide (H_2O_2) was used to establish an oxidative cell injury model. Our results revealed that myricitrin significantly improved some osteogenic markers in these cells. Myricitrin decreased lipid production and reduced *peroxisome proliferator-activated receptor gamma-2* (*PPARγ2*) expression in hBMSCs. Moreover, myricitrin reduced the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and IL-6 and partially suppressed ROS production. *In vivo*, we established a murine ovariectomized (OVX) osteoporosis model. Our results demonstrated that myricitrin supplementation reduced serum malondialdehyde (MDA) activity and increased reduced glutathione (GSH) activity. Importantly, it ameliorated the micro-architecture of trabecular bones in the 4th lumbar vertebrae (L4) and distal femur. Taken together, these results indicated that the protective effects of myricitrin against osteoporosis are linked to a reduction in ROS and bone-resorbing cytokines, suggesting that myricitrin may be useful in bone metabolism diseases, particularly osteoporosis.

Introduction

Osteoporosis is defined as a reduction in bone mass and the disruption of bone micro-architecture, which results in a decrease in bone strength and an increase in fracture risk (Poole and Compston, 2006). Oxidative stress is an imbalance between the excessive production of reactive oxygen species and insufficient antioxidant defence mechanisms (Sanchez-Rodriguez et al., 2007). Oxidative stress results in oxidative damage in all cellular components, including proteins, lipids and nucleic acids. On the basis of epidemiological studies in humans and mechanistic studies in animal models, oxidative damage has been shown to be a crucial pathogenic factor of osteoporosis (Manolagas, 2010). For example, osteoporotic postmenopausal women exhibit a decrease in bone mineral density that is related to higher oxidation of plasma lipids (Sendur et al., 2009) and lower superoxide dismutase and catalase efficacy (Ozgocmen et al., 2007; Witko-Sarsat et al., 1996). In rat femurs, ovariectomy results in oxidative stress and decreases the capacity of antioxidant defence mechanisms (Muthusami et al., 2005). Thus, oxidative stress might serve as a major contributor to postmenopausal osteoporosis (Sendur et al., 2009).

Bone remodeling occurs throughout life via synthesis of bone matrix through the action of two major cell types: osteoblasts and osteoclasts (Boyle et al., 2003). Osteoblasts are responsible for bone formation, while osteoclasts are in charge of bone resorption (Soltanoff et al., 2009; Huang et al., 2007). The proper functioning of these two cell types is necessary for the maintenance of bone mass as well as bone mineral density.

hBMSCs have the capacity to multi-differentiate into osteoblasts, adipocytes, chondrocytes, muscle cells, and neuronal cells. Moreover, they exhibit a self-renewal property (Pittenger et al., 1999). However, an imbalance between osteogenic and adipogenic differentiation has been reported to correlate with aging or diseases such as osteoporosis. In aging patients, particularly those with osteoporosis, decreased bone mineral density was accompanied by a reduction in osteoblasts but increased adipocytes, indicating that it is essential to balance adipogenic and osteogenic differentiation to maintain bone micro-architecture and volume (Jung et al., 2011).

Significant changes exist in bone turnover in postmenopausal osteoporosis: an increase in bone resorption (or resorption remains unchanged) and a decrease in bone formation, which result in bone loss

* Corresponding authors.
** Corresponding author. Tel./fax: +86 29 84771013.
E-mail address: liujianhq@sina.com (J. Liu).
[1] These authors contributed equally to the work.

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(Isomura et al., 2004). Thus, natural substances that possess antioxidant activity might modulate the activity of these two cell types in the maintenance of bone structure and volume.

Myricitrin, a botanical flavonol glycoside, is abundant in the fruits, branches, bark, and leaves of *Myrica cerifera* and other plants. As an important supplement, myricitrin is used in functional foods, cosmetics, and medicines contributing to its active properties. Previous reports have shown that myricitrin exhibits anti-nociceptive (Meotti et al., 2006a,b), anti-inflammatory (Wang et al., 2010), anti-allodynia (Meotti et al., 2006a,b), neuroprotective (Wang et al., 2013) and antioxidative (Moser, 2008) properties. Acrylamide-induced cytotoxicity is closely associated with oxidative stress in Caco-2 cells. Interestingly, myricitrin was able to suppress acrylamide toxicity by inhibiting reactive oxygen species production (Chen et al., 2013b).

However, few studies have investigated whether myricitrin could exhibit protective effects against osteoporosis via the reduction of reactive oxygen species and bone-resorbing cytokines. Here, we designed a H_2O_2-induced oxidative cell injury model in MC3T3-E1 cells and hBMSCs *in vitro*. Moreover, a murine ovariectomized model was generated *in vivo* to investigate the potential anti-osteoporosis effects of myricitrin and its underlying mechanisms.

Materials and methods

Materials. Myricitrin (purity > 98%, dissolved in distilled water) was purchased from Shanghai Tauto Biotech Co., Ltd. (China). The ALP activity assay kit was obtained from GENMED Scientific Inc. (USA). The RANKL and IL-6 ELISA assay kits were obtained from R&D Systems Inc. (Minneapolis, MN, USA). The total RNA kit was obtained from OMEGA. Oligonucleotide primers were synthesized by TaKaRa Biotechnology (Dalian, China). The ROS Assay kit, GSH and MDA Assay kits were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). All other reagents were of analytical grade.

Cell culture and differentiation. Murine MC3T3-E1 cells were cultured in α-MEM with 10% heat-inactivated foetal bovine serum (FBS) and 100 U/ml penicillin and 100 mg/ml streptomycin in a condition of 5% CO_2 and 37 °C. Six bone marrow samples were obtained for this research. Bone marrow (5 mL) aspirates were extracted from the posterior iliac crest of 6 healthy volunteers, aged 40–65 years (3 females and 3 males). Official approval by the Ethics in Clinical Research Committee of the First Affiliated Hospital of the Fourth Military Medical University (permission code 20120405-5) was obtained for the experimental procedures on humans and human cells. All of the donors were patients undergoing bone graft surgery, however, these patients did not suffer from any particular diseases and were otherwise healthy. hBMSCs were isolated using Percoll density gradient centrifugation and cultured in α-MEM with 15% heat-inactivated FBS and 100 U/ml penicillin and 100 mg/ml streptomycin in 5% CO_2 at 37 °C. Cells (Passage 3) were used in all of the following experiments. MC3T3-E1 cells and hBMSCs were incubated in an osteogenic induction medium (low-glucose DMEM administered with 10% FBS, 100 mM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid) or an adipogenic induction medium (low-glucose DMEM administered with 10% FBS, 0.5 mM isobutyl-methylxanthin, 1 μM dexamethasone, 200 μM indomethacin and 5 μM insulin). H_2O_2 served as an exogenous ROS source and N-acetyl-L-cysteine (NAC) was used as an ROS scavenger. After the cells had reached confluence, serum-free medium containing myricitrin was dissolved in distilled water and incubated for 24 h prior to treatment with 0.3 mM H_2O_2 for 24 h. For each experiment, myricitrin administration continued prior to H_2O_2 treatment. All of the experiments were performed in duplicate wells and repeated three times.

Assays of cell viability. In this study, MC3T3-E1 cells and hBMSCs were treated with different concentrations of myricitrin (0, 0.1, 1, 10 μM) for 24 and 72 h to measure the toxicity of myricitrin. MTT assays were performed as described (Chiang et al., 2012) and were used to examine cell viability. The absorbances of all wells were recorded on a microplate reader at 492 nm wavelength. The cell viability of the control group, which was not administered with myricitrin, was defined as 100%.

Alkaline phosphatase (ALP) activity assay. After an osteogenic induction for 6 days of MC3T3-E1 cells and 14 days of hBMSCs, they were incubated with serum-free medium containing myricitrin and/or H_2O_2 for 2 days. The ALP activity was measured as described (Wang et al., 2011). The cell monolayer was lysed and the lysate was centrifuged at 10,000 ×*g* for 5 min. The clear supernatant was used to test the ALP activity, which was determined using an ALP activity assay kit. Total protein concentrations were determined using the Bradford protein assay method.

Calcium deposition assay. After an osteogenic induction for 14 days of MC3T3-E1 cells and 21 days of hBMSCs, they were incubated with serum-free medium, which contained myricitrin and/or H_2O_2 for 2 days. When collected, these cells were used to test calcium deposition using Alizarin Red staining as previously described (Chen et al., 2013a). The cells were fixed with formalin for 20 min and stained with Alizarin Red S for 45 min at room temperature. To determine matrix calcification, unbound alizarin red was washed off with PBS. Next, the stain was solubilized with 10% cetylpyridinium chloride by shaking for 15 min. Absorbances of the released Alizarin Red S were recorded on a micro-plate reader at a wavelength of 562 nm.

Oil red O staining for adipogenesis. After an adipogenic induction for 14 days, hBMSCs were incubated with serum-free medium containing myricitrin and/or H_2O_2 for 2 days. The collected cells were tested for adipogenesis using Oil red O staining as previously described (Jung et al., 2011). The cells were rinsed with PBS, fixed in 3.7% formaldehyde for 20 min and stained with an Oil red O solution for 1 h. Next, the stained cells were imaged. For quantification of adipocytes, cell monolayers were washed extensively with water to remove unbound dye, and 1 ml of isopropyl alcohol was added to the culture dish. Adipocytes were quantified by counting red pixels in five random microscopic images per well using Adobe Photoshop software. Values were expressed as a percentage of the total pixels in each microscopic image.

Quantitative real-time PCR to test osteogenic and adipogenic differentiation. After an osteogenic induction for 6 days of MC3T3-E1 cells and an osteogenic or adipogenic induction for 14 days of hBMSCs, they were incubated with serum-free medium which contained myricitrin and/or H_2O_2 for 2 days. Total cellular RNA was extracted from these cells using Trizol reagent. Single-strand cDNA synthesis was performed

Table 1
Real-time PCR primers for amplification of specific mRNA.

MC3T3-E1		
Gene	Forward(5′-3′)	Reverse(5′-3′)
Alp	GCAGTATGAATTGAATCGGAACAAC	ATGGCCTGGTCCATCTCCAC
Colla1	GACATGTTCAGCTTTGTGGACCTC	GGGACCCTTAGGCCATTGTGTA
Ocn	ACCATCTTTCTGCTCACTCTGCT	CCTTATTGCCCTCCTGCTTG
β-Actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
hBMSC		
Gene	Forward(5′-3′)	Reverse(5′-3′)
Alp	CATGCTGAGTGACACAGACAAGAA	ACAGCAGACTGCGCCTGGTA
Colla1	GCTTGGTCCACTTGCTTGAAGA	GAGCATTGCCTTTGATTGCTG
Ocn	GGCAGCGAGGTAGTGAAGAGA	CTCCTGAAAGCCGATGTGG
PPARγ2	ATTCCATTCACAAGAACAGATCCAG	TTTATCTCCACAGACACGACATTCA
β-Actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA