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Promoting osteoblast differentiation by the flavanes from Huangshan Maofeng tea is linked to a reduction of oxidative stress

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ABSTRACT

Epidemiological evidence has shown an association between tea consumption and the prevention of bone loss in the elderly. Previous studies indicated that green tea exerted osteoprotective effect *in vivo*. This study aims to investigate the constituents in Huangshan Maofeng tea and systemically evaluate their antioxidative and osteogenic effects *in vitro*. Five flavanes, isolated from Huangshan Maofeng tea, showed effects on proliferation of osteoblastic cells and ameliorated H_2O_2-induced C2C12 mouse myoblast cell apoptosis at 3.125–50 μg/ml. (−)-Epicatechin observably increased alkaline phosphatase (ALP) activity and hydroxyproline content. (−)-Epiafzelechin at 25 μg/ml significantly increased the area of mineralized bone nodules. The activities of flavanes in promoting osteblastic proliferation and differentiation are positively correlated with activities in protecting against apoptosis in C2C12 cells. It indicates that anti-osteoporosis effect of these flavanes may be linked to their antioxidative activity. The observed effects of these flavanes suggest that these flavanes may have beneficial effects on bone health.

Introduction

Osteoporosis is a disease characterized by the loss of bone mass and degeneration of bone microstructure, resulting in an increased risk of fracture. Osteoporosis, called postmenopausal osteoporosis, is common in women after menopause (Ozgocmen et al., 2007). It may also develop in men, especially in the aged man, which is called age-related bone loss (Overton and Basu, 1999). Osteoporosis may significantly affect life expectancy and quality of life in humans. Bone integrity requires a tight coupling between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts (Riggs and Melton, 1992). During bone formation, osteoblasts undergo a cascade of complex events that might include three phases: proliferation, osteogenic differentiation, and mineralization of extracellular matrix (Zhang et al., 2008). The phenotype of mature osteoblasts is characterized by their ability to synthesize and secrete molecules of the extracellular matrix. Osteoblasts regulate them mineralization of the formed matrix by producing alkaline phosphatase (ALP) (Aubin, 1998). This enzyme hydrolyzes phosphate esters to increase the local phosphate concentration and enhance mineralization of the extracellular matrix (Lian et al., 1999). One of the characteristics of a mature osteoblast phenotype is the ability of the cells to synthesize ALP, which is considered an early marker of osteoblast differentiation (Tong et al., 1999). Collagen type I is an marker of osteogenic mature, and bone is a matrix with collagen type I (Sakano et al., 1993).

Oxidative stress, resulting from excessive formation of reactive oxygen species (ROS) or dysfunction of antioxidant defense system, represents a major cause of age-associated pathological conditions including aging (Linnane and Eastwood, 2006) and postmenopausal bone loss (Sendur et al., 2009). ROS are involved in osteogenesis including bone formation and resorption, which are associated with the aging process and may result in osteoporosis (Nohl, 1993; Basu et al., 2001; Muthusami et al., 2005; Isomura et al., 2004; Yalin et al., 2006). Oxidative stress is a pivotal pathogenic factor for age-related bone loss in mice, leading to an increase in osteoblast and osteocyte apoptosis and a decrease in osteoblast number and the rate of bone formation. Oxidative stress is involved in the osteoporosis from analysis of bone metabolism in iron-overloaded rats (Isomura et al., 2004). On the other hand, evidence suggests that ROS is also involved in bone resorption with a direct contribution of osteoclast-generated superoxide to bone degradation (Yang et al., 2001; Sontakke and Tare, 2002). Oxidative stress increases

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differentiation and function of osteoclasts (Sontakke and Tare, 2002), and inhibits osteoblastic differentiation (Mody et al., 2001; Fatokun et al., 2008).

Camellia sinensis, of the genus *Camellia*, is an evergreen plant that grows mainly in tropical and subtropical climates. Its leaves and leaf buds are used to produce the popular beverage tea. Huangshan Maofeng tea is a green tea produced in Anhui province of China. The tea is grown near Huangshan Mountain at altitudes of over 700 m. What distinguishes Huangshan Maofeng tea from other Maofeng teas is the color of its leaves. The tea liquor is jade green in color and has a light flowery fragrance. The tea is one of the most famous teas in China and can always be found on the China famous tea list. Green tea, unlike the preparation of black tea in which the leaves are fermented and oxidized, is produced by steaming the leaves, which preserves their polyphenolic content. The polyphenols comprise about 30–40% of the solid extract of leaves and are mostly categorized as catechins (Brown, 1999). Epidemiological studies have shown an association between a reduced risk of osteoporosis and the consumption of tea, including green tea (Shen et al., 2009a; Park et al., 2012; Shen et al., 2009b, 2010a,b; Hamdi Kara et al., 2007; Muraki et al., 2007; Hegarty et al., 2000; Wu et al., 2002; Devine et al., 2007; Yang and Landau, 2000). In previous study, green tea and green tea polyphenols (GTPs) benefit for bone health in ovariectomized (OVX) mice and old mice (Shen et al., 2009a; Shao et al., 2011; Shen et al., 2010a,b, 2009b, 2011a; Nakamura et al., 2010; Shen et al., 2011b). These evidences showed the therapeutic effects of the green tea on prevention of osteoporosis, however, most of these studies were focused on the crude extract or green tea polyphenols.

The present study is designed to investigate the chemical components of Huangshan Maofeng tea and evaluate potential osteogenic effects of these five flavanes on the proliferation, differentiation and mineralization of osteoblastic cells. To clarify the underlying mechanisms of flavanes, we investigated whether protection against osteoporosis by flavanes is linked to a reduction of oxidative stress.

Materials and methods

General

Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. NMR spectra were recorded on a Bruker DPX-400 spectrometer (400 MHz for ^{1}H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts were showed as the δ-value with reference to tetramethylsilane (TMS) as an internal standard. And ESI-MS data were obtained on an Agilent 1200 HPLC/6410B TripleQuad mass spectrometer. Diaion D-101 macroporus resin was the product of Xi'an Lanxiao Resin Corporation Ltd. (Xi'an, China). Sephadex LH-20 (Pharmacia, Sweden), silica gel (Qingdao Ocean Chemical Co., Ltd., Qingdao, China), and octadecylsilanized (ODS) silica gel (Macherey-Nagel, Duren, Germany) were used for column chromatography. TLC was carried out on Silica gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany), and RP-18 F_{254} (0.25 mm, Merck, Darmstadt, Germany) plates, and spots were visualized by spraying with 15% H_2SO_4 followed by heating. HPLC was performed using an octadecylsilanized (ODS) silica gel column (XTerra 10 μm, 19 mm × 250 mm, Waters). RPMI-1640 medium, fetal bovine serum (FBS), and trypsin-EDTA solution (1×) were obtained from Hyclone (Logan, UT). Annexin-V/PI Apoptosis Detection Kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Hydroxyproline Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were analytical or HPLC grade and obtained from Shanghai Chemical Reagents Co., Ltd. (Shanghai, China).

Extraction and isolation

Huangshan Maofeng tea were purchased in Huangshan, Anhui Province, China, and subjected to taxonomic identification with Voucher specimens (No. 120708) deposited at the herbarium of Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical College, China. The tea (2.5 kg) was extracted three times with 70% ethanol. The solvent was removed under vacuum to yield the crude extract (700 g). A suspension of the extract in H_2O was centrifuged and then applied to a D-101 macroresin column (80 mm × 1300 mm) and eluted with H_2O (10 l), 10% ethanol (10 l), 30% ethanol (10 l), 50% ethanol (10 l), 70% ethanol (10 l), and 95% ethanol (10 l) successively. Each eluent was concentrated and dried to yield 220.3 g, 100.0 g, 89.0 g, 150.0 g, 67.6 g, 6.5 g of dried elutes, respectively.

Bioactivity-guided fractionation was used for the isolation work. On the basis of the bioactive results of the extracts, the 70% ethanol eluent with the most potential activity was fractionated over a silica gel (200–300 mesh) column eluting with a gradually amount of MeOH in $CHCl_3$ to give 15 fractions. The $CHCl_3$-MeOH (25:1) elution was further purified by silica gel column, Sephadex LH-20, together with preparative HPLC, and got compounds **4** (152.33 mg), and **5** (70.56 mg), respectively. The $CHCl_3$-MeOH (15:1) elution was subjected to an octadecylsilanized (ODS) silica gel column, followed by a preparative HPLC with 20% methanol (containing 0.1% CF_3COOH, pH 3.0) to give compounds **1** (65.56) and **2** (41.34 mg). The $CHCl_3$-MeOH (15:1) elution was separated over an ODS column, followed by preparative Rp-HPLC with 18.5% methanol (containing 0.1% CF_3COOH, pH 3.0) to give compound **3** (33.47 mg).

HPLC

HPLC was performed on Phenomenex C_{18} column (Ø 250 mm × 4.6 mm) in Agilent series 1100 (USA) to analyze the compounds in Huangshan Maofeng tea under following conditions: mobile phase: (A) H_2O and 0.1% CF_3COOH, (B) MeOH; elution program: linear gradient from 10% B to 30% B in 30 min, 30% B to 50% B in 20 min, 50% B to 80% B in 10 min and then 100% B maintained for 20 min; flow rate: 0.80 ml/min; detection wavelength: 254 nm; injection volume: 10 μl; and oven temperature: 24 °C.

Culture of rat osteoblast cells

Osteoblastic cells were enzymatically isolated from newborn rat calvaria by the method of Declercq et al. (Declercq et al., 2004). With some minor modifications. The bone pieces were digested sequentially in a trypsin II-S (25 mg)-collagenase IA (70 mg) in 15 ml PBS solution at 37 °C for 30 min. Rat osteoblastic cells obtained from the last three digestion steps were pooled and plated together in a T25 tissue culture dish at a concentration of 50,000 cells/cm^2. And cultured in DMEM supplemented with 10% FBS and 1% antibiotic (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humid atmosphere containing 5% CO_2. The medium was changed every 3 days to remove the non-adherent cells.

C2C12 cell culture

The C2C12 cell line was purchased from the American Type Culture Collection. Cells were cultured in basal medium constituted with Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) for incubation at 37 °C in a 5% CO_2 humidified atmosphere.