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RESEARCH ARTICLE

The Effect of the Major Components of Fructus Cnidii on Osteoblasts *In Vitro*

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Abstract
In traditional Chinese medicine, the cause of weak bones or bone loss is generally regarded as a result of kidney deficiency. Fructus Cnidii (FC), which is also known as She-Chuang-Zi, is a traditional herb that has been claimed to have kidney warming effects that invigorate Yang. In this study, we tried to determine the bone production-inducing effect of FC on osteoblastic cells *in vitro* using osthole, the main component of FC. Osteoblasts were isolated from neonatal Sprague-Dawley rat calvaria using the tissue piece culture method and treated with various concentrations of osthole ranging from 2.5 to 640 μg/mL, together with a blank control. Cell proliferation, alkaline phosphatase (ALP) activity, and bone nodules were measured. The cells were examined by hematoxylin-eosin staining, the Gomori Calcium-Cobalt method and immunofluorescent staining. The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (or MTT) assay, ALP assay, and bone nodule results indicated significantly enhanced osteoblastic proliferation and differentiation at concentrations of osthole ranging from 40 to 320 μg/mL. Concentrations lower than 40 μg/mL seemed less effective, and cytotoxicity to osteoblasts was observed at concentrations higher than 320 μg/mL. These results indicate that osthole is effective at inducing osteoblastic bone formation through the up-regulation of ALP activity. FC is a Chinese herb used to treat lumbar pain in clinical practice. Further studies concerning the effects and mechanism of osthole on osteoporotic patients and animals should be performed, as these studies may lead to the development of a drug treatment for osteoporosis in the future.

1. Introduction

Bone homeostasis requires balanced interactions between osteoblasts and osteoclasts. Osteoporosis, therefore, involves a reduction in skeletal mass due to an imbalance between bone resorption and bone formation [1]. Over the past 10 years, patients with osteoporosis have been treated with antiresorptive agents (estrogens, bisphosphonates, calcitonin), which reduce osteoclast bone resorption. These agents prevent bone from being broken down; allowing remodeling spaces to be filled, and improve bone strength and reduce fracture risk [2]. Recently, attention has turned away from osteoclast inhibition and towards agents including parathyroid hormone, growth hormone, insulin-like growth factor-1, bone morphogenetic protein-2, and vascular endothelial growth factor, which stimulate osteoblasts to form new bone and anabolic agents [3]. Therefore, anabolic agents with the ability to stimulate

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new bone formation and correct imbalances of trabecular microarchitecture, which are characteristic of established osteoporosis, are desirable [4]. Among these, *in vitro* studies, especially studies related to components isolated from Chinese herbal medicine, are gaining attention [5].

Osthole is one of the main components of the dried seeds from the Fructus Cnidii (FC) plant, which is also known by its traditional name She-Chuang-Zi (SCZ) [6]. FC is known for its effects including kidney warming, which is thought to invigorate Yang, and the clearance of heat and toxic materials from the body. FC is used in Traditional Chinese Medicine (TCM) in tonics and aphrodisiacs to treat impotence and lumbar pain in clinical practice [7,8]. Weak bones and bone loss are generally regarded to be caused by kidney deficiencies in TCM, but the bone-forming actions of FC on osteoporosis have rarely been reported. Osthole was reported to exhibit estrogen-like effects, preventing postmenopausal osteoporosis in ovariectomized rats [9], however, the biological effects of osthole on bone cells are relatively unknown. In this study, we determined the bone-inducing effects of osthole, the main component of FC, on osteoblast cells *in vitro*. We also describe the promoting activities of different concentrations of osthole on osteoblast cells.

2. Materials and Methods

2.1. Reagents and materials

The Chinese herb FC was obtained and its component osthole (7-methoxy-8-isopentenoxycoumarin, molecular weight: 244.29) was prepared by the National Institute for the Control of Pharmaceutical and Biological Products (product number: 110822-200406). Nine pre-experimentally-determined concentrations of osthole (0, 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 μg/mL) were diluted with DMEM-F12 (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin. All solutions used in cell cultures were sterilized by filtration through 0.2 μm Millipore filters and stored in a refrigerator at 4ºC until use. D-Hanks' solution, penicillin G, streptomycin, and all other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless stated otherwise.

2.2. Cell culture

Primary osteoblastic cells were prepared from neonatal (0–3 days old) Sprague-Dawley rats (CLEA Japan, Osaka, Japan). The rats were housed in a constant temperature-controlled environment (23ºC) with 60% relative humidity under a 12-hour light/dark cycle. The procedures involving the animals and their care were in full compliance with our institutional regulations and the current international laws and policies [10]. The rats were killed by being immersed in 70% alcohol for 10 minutes, and then the rat calvaria were excised, stripped of soft tissue, washed three times in D-Hanks' solution containing 0.25% trypsin, and cut into small pieces with a dissector at a volume of about 1 mm^3. The bone tissue pieces were homogeneously seeded at 1 cm intervals in flasks, which had been incubated in advance for 10 minutes with 1 mL of DMEM medium. After being kept inversely in an incubator at 37ºC in a 5% CO_2 fully humidified atmosphere for 2 hours, the flasks were then reversed to their normal position and 5 mL of DMEM/F12 containing 15% FBS and 1% penicillin-streptomycin was added in order to avoid adhered tissue pieces floating to the surface from the bottom. Flasks were then incubated continuously for 3 days in an incubator. Media was replaced every 3 days. After reaching confluence, the cells were passaged every 7 days.

2.3. Identification of osteoblast cells

Cellular morphology and growth were observed daily, after the first 3 days in primary culture, using an inverted microscope, whereupon images were obtained. We used methods similar to those described elsewhere for the identification of osteoblasts, in addition to hematoxylin-eosin staining [11]. Alkaline phosphatase (ALP) staining was also performed using the Gomori Calcium-Cobalt (Ca-Co) method. In brief, when the cells had reached confluence, after fixation with a solution of 95% alcohol for 10 minutes, the cells were incubated in an incubator at 37ºC for 4–6 hours. The cells were then stained with solutions of 2% cobalt nitrate and 1% ammonium sulfide in turn. After being air-dried, the slides were finally mounted and used for microscopy [12]. Immunocytochemistry, as described by others, was also performed to identify osteoblasts [13]. Briefly, cells were planted onto a coverslip. When 70% confluence had been reached, the coverslips were fixed using a 95% alcohol solution for 5 minutes. Mouse anti-collagen (type clone Col-1: 2456) was used as a primary antibody at a ratio 1:100, which was diluted in 0.3% Triton X-100 and 5% normal goat serum in phosphate buffered saline (PBS). The cells were incubated with the primary antibody present at 4ºC overnight before being washed in PBS. Anti-mouse IgG TRITC antibody produced in goats (Sigma-Aldrich, St-Louis, MO, USA) was used as the secondary reagent (1:100) after being diluted in 0.3% Triton X-100/PBS and incubated for 2 hours at 4ºC. After a final