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Ethyl-2, 5-dihydroxybenzoate displays dual activity by promoting osteoblast differentiation and inhibiting osteoclast differentiation

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ABSTRACT

The interplay between bone-forming osteoblasts and bone-resorbing osteoclasts is essential for balanced bone remodeling. In this study, we evaluate the ability of ethyl-2, 5-dihyrdoxybenzoate (E-2, 5-DHB) to affect both osteoblast and osteoclast differentiation for bone regeneration. Osteogenic differentiation of human mesenchymal stem cells (hMSCs) was quantified by measuring alkaline phosphatase (ALP) activity and calcium deposition. To evaluate osteoclast differentiation, we investigated the effect of E-2, 5-DHB on RANKL-activated osteoclastogenesis in RAW 264.7 cells. E-2, 5-DHB enhanced ALP activity and inhibited RAW 264.7 cell osteoclastogenesis in vitro. To assess the *in vivo* activity of E-2, 5-DHB, hMSCs were delivered subcutaneosuly alone or in combination with E-2, 5-DHB in an alginate gel into the backs of nude-mice. Histological and immunohistochemical evaluation showed significantly higher calcium deposition in the E-2, 5-DHB group. Osteocalcin (OCN) was highly expressed in cells implanted in the gels containing E-2, 5-DHB. Our results suggest that E-2, 5-DHB can effectively enhance osteoblast differentiation and inhibit osteoclast differentiation both in vitro and *in vivo*. Understanding the dual function of E-2, 5-DHB on osteoblast and osteoclast differentiation will aid in future development of E-2, 5-DHB as a material for bone tissue engineering.

1. Introduction

Bone remodeling plays a major role in the maintenance of skeletal mechanical integrity. This involves a well-coordinated balance between bone formation (by osteoblasts) and bone resorption (by osteoclasts) to guarantee that alterations in bone mass or quality do not occur after each remodeling cycle [1,2]. Certain pathological conditions can cause abnormal bone remodeling by creating an imbalance between resorption and formation, leading to development of bone disorders. A variety of factors, including menopause-associated hormonal changes, age-related factors, changes in physical activity, drugs, and secondary diseases can lead to development of various bone disorders in both women and men [3].

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of self-renewal and differentiation [4] into multiple lineages including osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic lineages [5]. During fracture processes, circulating progenitor cells are recruited to the site of injury to aid in heading [6,7] and MSC play a role in regenerating bones following bone injury. The native properties of stem and progenitor cells make them a common choice for stem cell transplantation, tissue engineering, and immunotherapies. In fact, techniques that utilize human bone marrow MSC differentiation to specific lineages in combination with biomimetic scaffolds provide the potential to engineer bone and cartilage when endogenous healing processes are not sufficient [8].

Alginate, a natural polysaccharide extracted from seaweed, is

Abbreviations: E-2, 5-DHB, Ethyl-2, 5-dihyrdoxybenzoate; hMSC, human mesenchymal stem cells; ALP, alkaline phosphatase; OCN, osteocalcin; TRAP, tartrate-resistant acid phosphatase; ARS, Alizarin Red S.

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one of the most common cell and drug delivery vehicles. When exposed to a divalent cation, alginate solutions quickly crosslink to form a hydrogel under mild and physiological conditions [9]. The gentle gelling conditions of alginate are superior for preserving the viability of incorporated cells and proteins, thereby minimizing detrimental effects compared to other systems. Alginate also has other merits for cell seeding, including good biocompatibility, high porosity, and an interconnected porous structure for nutrient and oxygen diffusion [10,11]. Sodium alginate (a crosslinked version of alginate that maintains viability due to diffusion limitations) is used in biomedical and biotech applications to encapsulate and immobilize a variety of cells [12,13].

Rubus coreanus total extracts have bone protecting-effects during postmenopausal osteoporosis in ovariectomized rats [14]. In a previous study we showed that ethyl-3, 4-dihydroxybenzoate (E-3, 4-DHB), a component of Rubus coreanus extract increases osteoinductive activity, by promoting osteoblast differentiation, and decreases bone resorption, by inhibiting osteoclast differentiation [15]. E-3, 4-DHB has an ethyl benzoate structure with hydroxyl groups on the 3 and 4 carbons of the benzene ring. Ethyl-2, 5-dihydroxybenzoate (E-2, 5-DHB), however, is another component of the Rubus coreanus extract that has a different ethyl benzoate structure with hydroxyl groups on the 2 and 5 carbons of the benzene ring (Fig. 1A). There two materials are common component of Rubus coreanus extract, but structurally different materials.

In the present study, we evaluated the ability of E-2, 5-DHB to modulate osteoblast and osteoclast differentiation to promote bone regeneration. Osteoblast and osteoclast differentiation were assessed in vitro in hMSCs and in a murine preosteoclast cell line. To assess osteoblast and osteoclast differentiation *in vivo*, hMSCs, alone or in combination with E-2, 5-DHB, were delivered in an alginate gel subcutaneously into nude mice. Histological and immunohistochemical evaluations of the tissue were used to assess differentiation. Our results show that E-2, 5-DHB has dual affects on both osteoblast and osteoclast differentiation and suggest that it could be a potential agent used to improve bone tissue engineering in the future.

2. Materials and methods

2.1. Materials and animals

E-2, 5-DHB was supplied by CellSafe (Suwon, Korea) and dissolved in ethanol. Sodium alginate, calcium chloride, dexamethasone, β-glycerol phosphate, and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Osteogenic media was composed of dexamethasone (10 nM), β-glycerol phosphate (10 mM), and ascorbic acid (50 μg/ml).

Balb/c nude mice (6-wks-old) were purchased from Orient bio (Korea). All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit # 2011-0107). All mice were maintained in a specific pathogen-free facility at the YLARC.

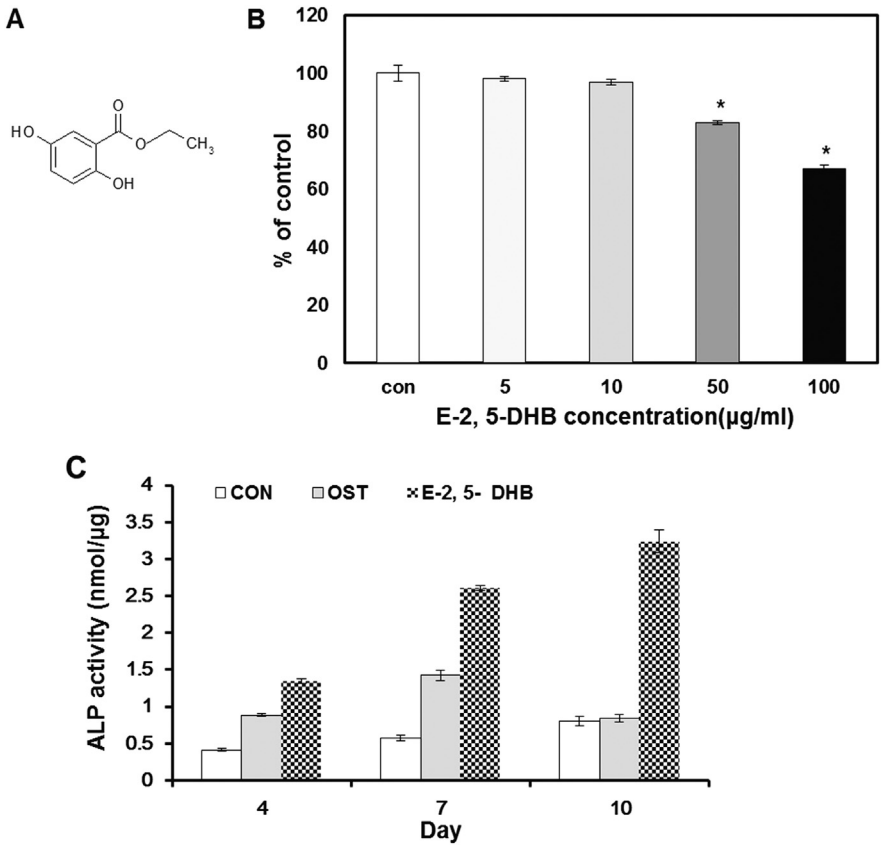

Fig. 1. E-2, 5-DHB displays dose-dependent cytotoxicity. The structure of E-2, 5-DHB (A). The effect of E-2, 5-DHB on cytotoxicity was measured in L-929 cells by MTT assay (B). Cell number on a given day is expressed as a percent of the control (non-treatment, day 1). E-2, 5-DHB stimulates hMSC differentiation in culture, as measured by ALP activity (C). hMSCs were treated with E-2, 5-DHB during differentiation (days 4–10). ALP activity was measured via colorimetric assay. Data are shown as means ± SD (n = 3, * $p < 0.05$ compared with non-treated cells).