Fermented Sargassum fulvellum-Derived Vitamin K Promotes Anti-Inflammatory and Osteogenic Activities

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모자반 발효물 비타민K의 항염과 골대사 활성효능

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Abstract

Vitamin K (VitK) is a vital nutrient that is newly recognized to support bone and cardiovascular health. As a nutraceutical, VitK is produced via plant extraction and bacterial fermentation. This study examined the potential anti-inflammatory and osteogenic benefits of VitK, i.e., VitK1 (phyl-loquinone; PK) and VitK2 (menaquinone; MKs), derived from Sargassum fulvellum fermented by Lactococcus lactis and Leuconostoc mesenteroides (SfLlLm) using lipopolysaccharide (LPS)-induced Raw264.7, MC3T3-E1 cells, and ovariectomized (OVX) mice. MK4, MK7, and MK9, as well as PK, were effectively acquired from SfLlLm and analyzed. SfLlLm_VitK reduced levels of proinflam-matory cytokine in LPS-induced Raw264.7 cells and induced osteogenesis regulating factor in MC3T3-E1 cells. In OVX mice, SfLlLm feeding reduced plasma levels of alkaline phosphatase, phosphate, and pro-collagen type I alpha 2 gene (pro-Col1a2) while elevating cancellous bone volume and trabecular number. Accordingly, SfLlLm, comprising MKs, may be a candidate for preventing and treating immune and bone diseases.

1. Introduction

Algae form the basis of the food chain and cover more than 70% of the world's total living mass. Sargassum fulvellum (S. fulvellum) is popular and inexpensive edible brown seaweed enriched in nutrients, such as carbohydrates, lipids, proteins, vitamins, and minerals, and is also a source of several health-promoting components, including fuco-xanthin, phlorotannins, and polysaccharides [1]. Currently, owing to the growing demand for vitamins as market leaders for high-value bioproducts, algae-derived vitamins are gaining traction for applications in food, feed, cosmetic, chemical, and pharmaceutical industries. Vitamins, essential micro-nutrients for life, are either not or minimally synthe-sized in animals and humans, thus necessitating continuous assimilation through diet intake of plants, fruits, or seeds [2]. Most vitamins are synthesized by photosynthetic or-ganisms, whereas vitamins B and K are bioaccumulated through the diet and are mainly produced by bacteria [3]. For use as nutraceutical and dietary supplements, large-scale vitamin production is currently accomplished through chemical synthesis, extraction from natural sources, and microbial fermentation [4]. Given the health benefits of vitamin K (VitK) and increasing consumer preference for natural products, there is a clear need to identify sustainable and cost-effective methods for producing natural-derived VitK. Nota-bly, VitK exists mainly in two natural forms: VitK1 (phylloquinone; PK), which is present in plants and algae, and VitK2 (menaquinone; MKn or MKs), which is present in animal foods (MK4) or synthesized by microorganisms [5](MK5 to MK13; Akbari and Ra-souli-Ghahroudi 2018). MKs, including MK7 and MK9, are bacterial products found in fermented foods, such as natto and chenggukjang produced by Bacillus subtilis and cheese by Lactoccocus ssp [6-8]. PK and MKs play multifunctional roles in a wide range of

biolog-ical activities, including blood coagulation, bone maintenance, prevention of arterial hardening, neuroprotection, and modulation of inflammation [9-12]. Recently, the im-portance of vitamin K, calcium, and vitamin D has been highlighted in the prevention and treatment of osteoporosis [13,14](Villa et al., 2017; Capozzi et al., 2020).

This study focused on long-chain MKs, given that they are produced via algae- and LAB-based fermentation and could exert immune and bone health benefits compared with algae-based VitK forms. The objective of the current study was to provide insights into the natural MK production capacities of L. lactis and L. mesenteroides and the influence of S. fulvellum nutrients, including PK, on MK production. Initially, the MK forms and quantity produced by the co-culture of L. lactis and L. mesenteroides with S. fulvellum were deter-mined. Then, the anti-inflammatory and osteogenic effects of the obtained VitK were ana-lyzed in LPS-treated RAW264.7 cells, MC3T3-E1 preosteoblasts, and ovariectomized (OVX) mice.

2. Materials and Methods

2.1 . Preparation of S. fulvellum Extract and Fermentation

S. fulvellum was purchased from Parajeju (http://www.parajeju.com), dried, ground, and autoclaved to prepare 5% Sf media (w/v in distilled water; Sf) before fermentation. In previous studies, LAB L. lactis (Ll, KCCM12759P) and L. mesenteroides (Lm, KCCM12756P) were isolated from the East Sea of the Republic of Korea and selected to produce mena-quinone (VitK2; MKs) using genomic PCR using menA ~ G gene primers [16,17].

2.2 VitK Analysis

The extraction and analysis of VitK1 and MKs in Sf and SfLlLm were performed us-ing an established procedure with modifications [22].

2.3 Antioxidant Analysis

First, the free radical scavenging activity of Sf and SfLlLm was determined using the DPPH assay (Sigma, USA). In 96-well microplates, 30 µL of sample and 70 µL DPPH solu-tion (0.2 mM in methanol) were added and reacted for 30 min under dark conditions. After the reaction, the optical density (OD) was measured at 517 nm using a microplate reader (Thermo Fisher Scientific). The percentage inhibition was calculated as follows:

DPPH radical scavenging activity (%) = {1 − (OD sample/OD blind)} × 100

2.4 Cell Culture and Differentiation

RAW264.7 cells, a macrophage cell line, were purchased from Korean Cell Line Bank (KCLB, Korea) and cultured in Dulbecco Modified Eagle Medium (Gibco, USA), supple-mented with 10% fetal bovine serum (FBS; v/v; Gibco, USA) and 1% penicil-lin/streptomycin (v/v) at 37℃ in a humidified incubator with 5% CO2 atmosphere. Cytotoxicity was determined using the Cytotoxicity LDH Assay Kit-WST (Dojindo, Korea), as described in the manufacturer's protocol. The NO level in the cell culture medium was detected using a NO assay kit (Dogendo, Ko-rea) at 540 nm. ALP activity was determined using an Alkaline Phosphatase Detection Kit (Merck, USA).

2.5 OVX Animal Model

First, 20 C57BL/6 female mice (8-week-old; Orient Bio Inc., Korea) were randomly di-vided into four groups (n=5 mice/group): sham (control; CONT), OVX (negative control; nCONT), OVX + 10 % Sf, and OVX + 10 % SfLlLm group. After induction of anesthesia us-ing tribromoethanol, the groups were separately subjected to sham surgery without ovari-ectomy or bilateral ovariectomy to induce osteoporosis.

2.6 Data Analysis

Statistical significance was determined using one-way ANOVA with GraphPad Prism 8.0 (GraphPad Software, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1 VitK Identification in Sf and SfLlLm and the

Antioxidant Effect

he Sf- and SfLlLm-derived PK and MKs used for in vitro and in vivo experiments were prepared and identified as shown in Figure 1A. To develop fermented S. fulvellum with high PK and MK contents, L. lactis and L. mesenteroides strains with male genes were co-inoculated in 5% Sf media for MK production (Figure 1A; [17]. To enhance the MK con-tent of Sf with natural PK, Sf was fermented with both LAB at 30℃ after 24 h (SfLlLm) and extracted with isopropanol; then, the PK and MK contents were measured.). These results suggest that SfLlLm may help prevent or treat osteoporosis and bone in-flammatory diseases.

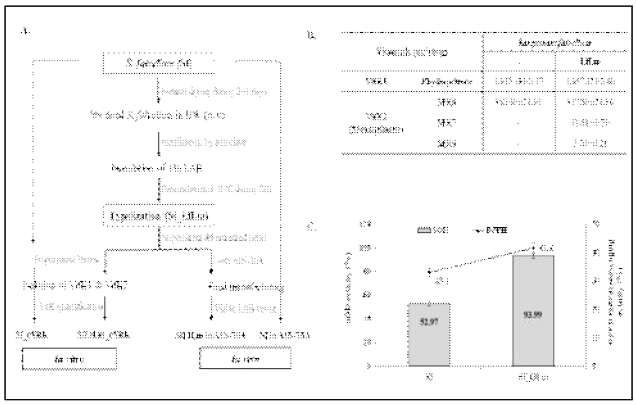

Fig. 1. Preparation, antioxidant assessment, and quantification of vitamin K from Sf and SfLlLm. (A) Flowchart of sample preparation from S. fulvellum for in vitro and in vivo assessments.

3.2 Inflammatory Effect in LPS-induced Raw264.7 cells

To determine the effects of Sf and SfLlLm with complex VitK (PK and MKs) on cell vi-ability, Raw264.7 macrophage cells were treated with the isolated Sf_cVitK and SfLlLm_cVitK (Figure 1A and 1B) at different concentrations ranging from 0, 280, 560, and 840 ng/mL for 24 h. These data verified that SfLlLm_cVitK could regulate the LPS-induced production of NO and cytokines when compared with Sf_cVitK. Reportedly, MKs can downregulate basal and cytokine-induced NF-κB activation in human and murine monocytic cell lines, there-by preventing bone resorption [27]; these effects are triggered by the side chains of MKs but not by PK side chains [28]. These findings support the possibility that MKs produced from SfLlLm can suppress inflammation in LPS-induced Raw264.7 cells.

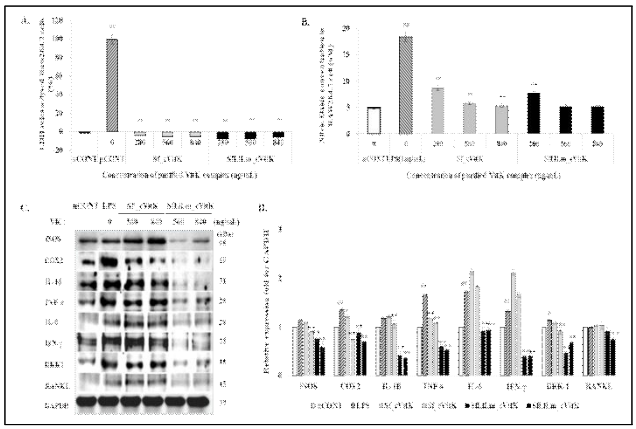

Fig. 2. Anti-inflammatory effects of VitK isolated from nonfermented and fermented S. fulvellum in LPS-stimulated Raw264.7 cells.

3.3 Effects of Osteogenic Activation and Mineralization in MC3T3-E1 Cells

The effects of Sf_cVitK and SfLlLm_cVitK on the cytotoxicity, differentiation, and mineralization in MC3T3-E1 preosteoblastic cells were determined. The normalized ALP concentration in the medium indicated normal stimulation of osteo-blast differentiation or extracellular matrix mineralization in Sf_cVitK- or SfLlLm_cVitK-treated MC3T3-E1 cells. These results suggest that MK4 present in Sf and SfLlLm likely stimulates the differentia-tion and mineralization of preosteogenic MC3T3-E1 cells. Additionally, MK7, as an MK4 precursor, may be superior to MK4 itself to achieve MK4-specific physiological effects. VitK (PK and MKs) produced by the fermentation of Sf by LAB were consistent with those obtained in previous studies [32], correlating with MKs capable of enhancing osteoblast differentiation and mineralization.

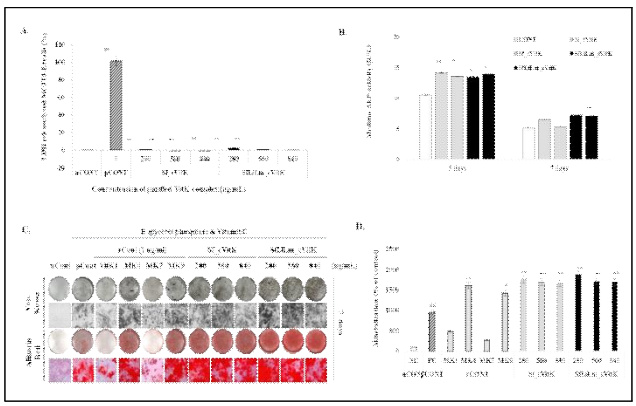

Fig. 3. Preosteoblast differentiation and mineralization effects of VitK isolated from Sf and SfLlLm in MC3T3-E1 cells.

3.3 Effects of Sf and SfLlLm in OVX Mice

Next, the preventive effect of fermented sargassum-derived VitK (PK and MKs) on OVX-induced bone loss was examined in 8-week-old female mice. Experimental mice were fed a normal diet (AIN-76A) and AIN-76A comprising 10% Sf and 10% SfLlLm (Sf and SfLlLm) for 8 weeks, starting two weeks before OVX or sham surgery (Figure 4A). After 8 weeks, the body weight of mice in the OVX group fed a normal diet (nCONT) was significantly higher than that of mice in the sham group (CONT) and mice in the OVX groups fed Sf and SfLlLm diets ($p<0.01$; Figure 4B). These findings support the possibility that VitK, including MKs from SfLlLm produced by LAB, stimulates osteoblasts in OVX mice.

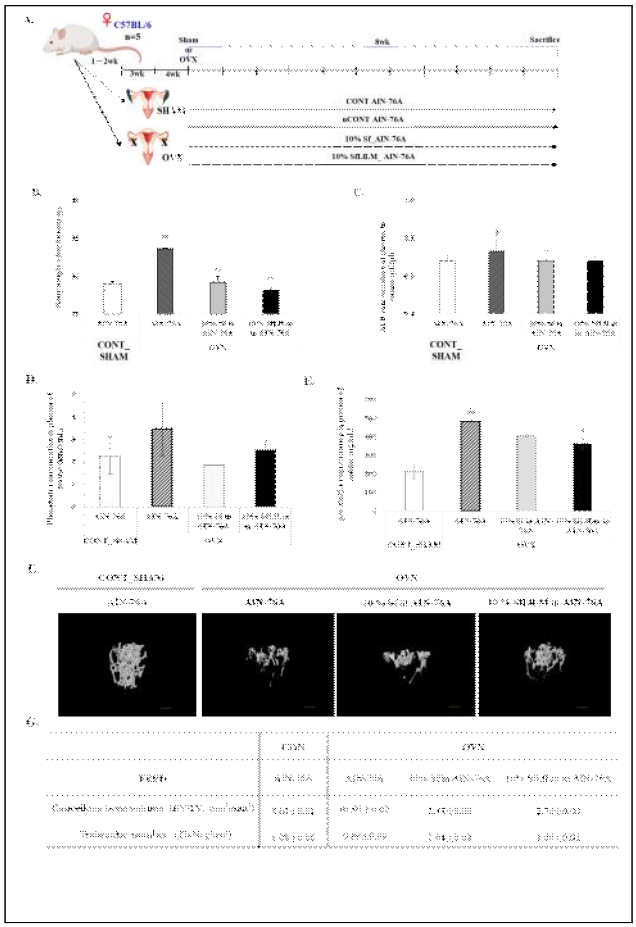

Fig. 4. Osteogenic effect in ovariectomized (OVX) mouse.

4. Conclusions

In conclusion, the study demonstrated that the potential anti-inflammatory and os-teogenic benefits of VitK (PK, MK4, MK7, and MK9) derived from S.fulvellum fermented by L. lactis KCCM12759P and L. mesenteroides KCCM12756P using LPS-induced Raw264.7, MC3T3-E1 cells, and OVX mice. Therefore, SfLlLm including VitK are a highly effective source of natural compounds for inflammation relief and bone health, where treatment op-tions are limited and can have positive effects on the food and pharmaceutical industries.

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