Effects of Brown Rice Extract Treated with *Lactobacillus sakei* Wikim001 on Osteoblast Differentiation and Osteoclast Formation

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ABSTRACT: Phytic acid (*myo*-inositol hexakisphosphate) or phytate is considered an anti-nutrient due to the formation of precipitated complexes that strongly reduces the absorption of essential dietary minerals. In this study, brown rice with reduced phytate was made by inoculation with *Lactobacillus sakei* Wikim001 having high phytase activity. The effects of brown rice extract treated with *L. sakei* Wikim001 (BR-WK) on osteoblast differentiation and osteoclast formation were investigated. The proliferation of SaOS-2 cells was measured by the MTT assay. Treatment with BR-WK increased cell proliferation by 136% at a concentration of 100 μg/mL. The Alkaline phosphate activity in SaOS-2 cells was 129% higher when BR-WK was processed at a concentration of 100 μg/mL. The proliferation of bone marrow macrophages decreased by nearly 60% in response to treatment with BR-WK. In addition, BR-WK reduced the number of tartrate-resistant acid phosphatase-positive (TRAP$^+$) multinucleated cells from bone marrow macrophages. These results indicate that BR-WK stimulates bone formation through its positive action on osteoblast differentiation and function and furthermore, decreases osteoclast differentiation.

Keywords: brown rice, *Lactobacillus sakei*, phytate, osteoblast, osteoclast

INTRODUCTION

As consumers' awareness and demand about healthier foods have increased in recent years, the consumption of brown rice has also increased (1). Brown rice has higher concentrations of dietary fiber, fat, phytic acid, and total polyphenols compared to white rice (2,3). However, phytic acid (*myo*-inositol hexakisphosphate) or phytate is considered an anti-nutrient due to the formation of precipitated complexes that strongly reduce the absorption of essential dietary minerals (4). Phytase is a generic term used to describe an enzyme that hydrolyses phosphomonoester bonds from phytic acid (5). Phytase [*myo*-inositol hexakis (dihydrogen-phosphate) phosphohydrolase, EC 3.1.3.8] catalyses the hydrolysis of phytic acid into *myo*-inositol and phosphoric acid via penta- to mono-phosphate, thus decreasing or eliminating its anti-nutritional effect (6). Phytases are produced by a wide range of plants, bacteria, fungi and yeast (7,8).

In some lactic acid fermentations of corn, lentils and peas, a decrease in the content of phytates has been observed. Microbial phytases suitable for food fermentations could be obtained from lactic acid bacteria isolated from natural vegetable fermentations (9). After inoculating a whole wheat flour medium with the *Leuconostoc mesenteroides* subsp. *mesenteroides* strain 38 for 9 h, it was established that the degradation of phytic acid and the production of lactic acid lead to a greater Ca^{2+} and Mg^{2+} solubility than in the control medium (10).

Bone development in vertebrate animals is maintained by two coordinated actions, osteoblast (bone formation) and osteoclast (bone resorption). In fact, many bone disorders reflect imbalanced activities of osteoblast and osteoclast, leading to increased (osteopetrosis) and decreased (osteoporosis) bone mass (11). Calcium is an essential nutrient involved in most metabolic processes and the calcium phosphate salts provide mechanical rigidity to the bones and teeth, where 99% of the body's calcium resides (12). Calcium affects bone diseases. In particular, the lack of calcium in postmenopausal women increases bone loss leading to osteoporosis (13). For the prevention and treatment of osteoporosis, hormone replacement therapy has been widely used; however, many side effects have been reported. Thus, prevention and treatment of osteoporosis via food intake studies are currently underway (14). To investigate the effect of the

ingredients, such as sea mustard extract and soy extract, on bone formation, the inhibition of osteoclastic differentiation and stimulatory effect in cultured osteoblastic cells have been studied (15,16).

Our previous study revealed that *Lactobacillus sakei* Wikim001 isolated from kimchi has high phytase activity and is beneficial for reducing the phytate contents in brown rice (17). Thus, the effect of phytase-producing *L. sakei* Wikim001 treated brown rice extract (BR-WK) on osteoblast differentiation in Human SaOS-2 osteosarcoma cells as well as on osteoclast formation in mouse bone marrow cells was investigated.

MATERIALS AND METHODS

L. sakei culture and preparation of brown rice extracts
The *L. sakei* Wikim001 strain, which was isolated from *kimchi,* was inoculated into MRS broth (pH 6.5) at $30^{\circ}C$ for 24 h. Brown rice was produced in 2013, Gangjin, Jeollanam-do, Korea. Brown rice was inoculated with or without *L. sakei* Wikim001. The two samples of brown rice were incubated at $30^{\circ}C$ for 24 h. The dried samples were extracted with absolute ethanol (BR, extract of brown rice without *L. sakei* Wikim001; BR-WK, extract of brown rice treated with *L. sakei* Wikim001). Then, both brown rice extracts were stored at $-70^{\circ}C$ until use.

SaOS-2 cell culture
Human SaOS-2 osteosarcoma cells were obtained from a Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco). The cells were grown at $37^{\circ}C$ in 5% CO_2 and 95% air.

Bone marrow-derived macrophages preparation and osteoclast differentiation
Mouse bone marrow cells were obtained from femurs and tibias of a 6-week-old ICR mouse and were incubated in α-minimum essential medium (α-MEM) (Gibco) complete media containing 10% FBS, 100 U/mL penicillin in a 100 mm culture dish in the presence of a macrophage colony-stimulating factor (M-CSF, 30 ng/mL) for 3 days. Adherent cells, after the removal of non-adherent cells, were used as bone marrow macrophages (BMMs). To generate osteoclasts, BMMs (4×10^4 cells/well) were cultured for 4 days with M-CSF (30 ng/mL) as well with receptor activator of nuclear factor-κB ligand (RANKL, 100 ng/mL), in 24-well (1 mL/well) tissue culture dishes.

MTT colorimetric assay
Cell viability assay was carried out with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reduction was used in order to assess cell viability. SaOS-2 cells (1.0×10^4 cells/wells) were seeded in a 96-well plate with RPMI 1640 medium containing 10% FBS for 24 h. BMMs (1.0×10^4 cells/wells) were seeded in a 96-well plate with α-MEM containing 10% FBS for 24 h. Cells were added to various concentrations of brown rice extracts for 3 days, washed with PBS three times and treated with a medium containing 100 μg/mL of MTT for 4 h at $37^{\circ}C$, washed with PBS, and then solubilized in DMSO. The resulting intracellular purple formazan was quantified with a spectrophotometer by measuring the absorbance at 570 nm.

Alkaline phosphatase assay
The alkaline phosphate (ALP) activity was measured in order to observe the effects on osteoblast differentiation. After incubation with effectors, the SaOS-2 cells were washed twice with PBS, harvested with rubber policemen, and resuspended in PBS supplemented with PBS 1%, Triton X-100. The cell lysate was sonicated for 1 min. ALP activity was assayed by a spectrophotometric method using para-nitrophenyl phosphate as the substrate. The OD was measured at 405 nm with a spectrostar nano plate reader (BMG Labtech GmbH, Ortenberg, Germany). The results, normalized on a protein basis, were expressed as the percentages of the control. Protein determination was carried out with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA).

Tartrate-resistant acid phosphatase-positive (TRAP$^+$) assay
BMMs were seeded in 24-well plates at a concentration of 2.5×10^4 cells per well and pretreated with or without brown rice extracts. The cells were then stimulated with 100 ng/mL RANKL. After 6 days, a TRAP stain assay was performed in order to confirm cell differentiation rates. TRAP-positive cells were stained using a Leukocyte Acid Phosphate Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's procedure. TRAP-positive multinucleated cells (containing ≥3 nuclei) were then counted.

Statistics analysis
Each experiment was performed in triplicate, and the results were expressed as the mean±SD. Statistical evaluation was performed by analysis of variance (ANOVA) using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). Significant differences ($P<0.05$) between the means were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Both osteoblastic and osteoclastic cells regulate bone metabolism and are involved in the development of osteoporosis. Osteoblasts are bone forming cells located near the surface of the bone that produces cytokines (18). Osteoblasts, which arise from mesenchymal stem cell precursors, undergo differentiation in response to various growth factors (19). The effects of brown rice
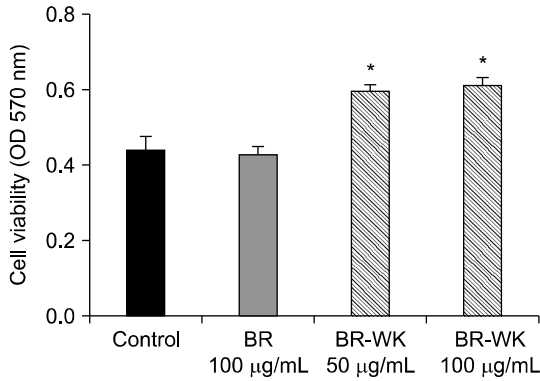
Fig. 1. Effects of brown rice extract treated with *Lactobacillus sakei* Wikim001 (BR-WK) on the viability of SaOS-2 cells by MTT assay. The cells were treated with brown rice extract (BR) and BR-WK for 4 days, then evaluated for viability by MTT assay. Each experiment was performed in triplicate, and the results were expressed as the means±SD. *$P<0.05$ vs. control.
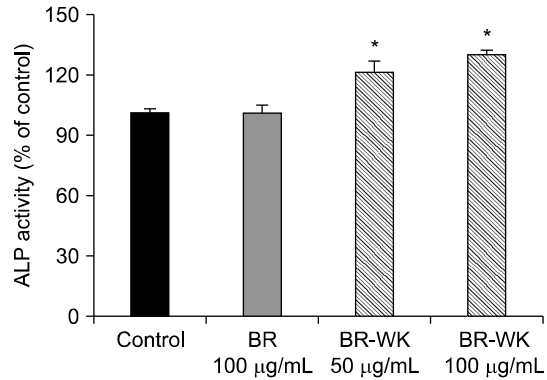
Fig. 2. Effects of brown rice extract treated with *Lactobacillus sakei* Wikim001 (BR-WK) on the alkaline phosphatase activity of SaOS-2 cells. The cells were treated with brown rice extract (BR) and BR-WK for 48 h. The data are presented as the means±SD. *$P<0.05$ vs. control.
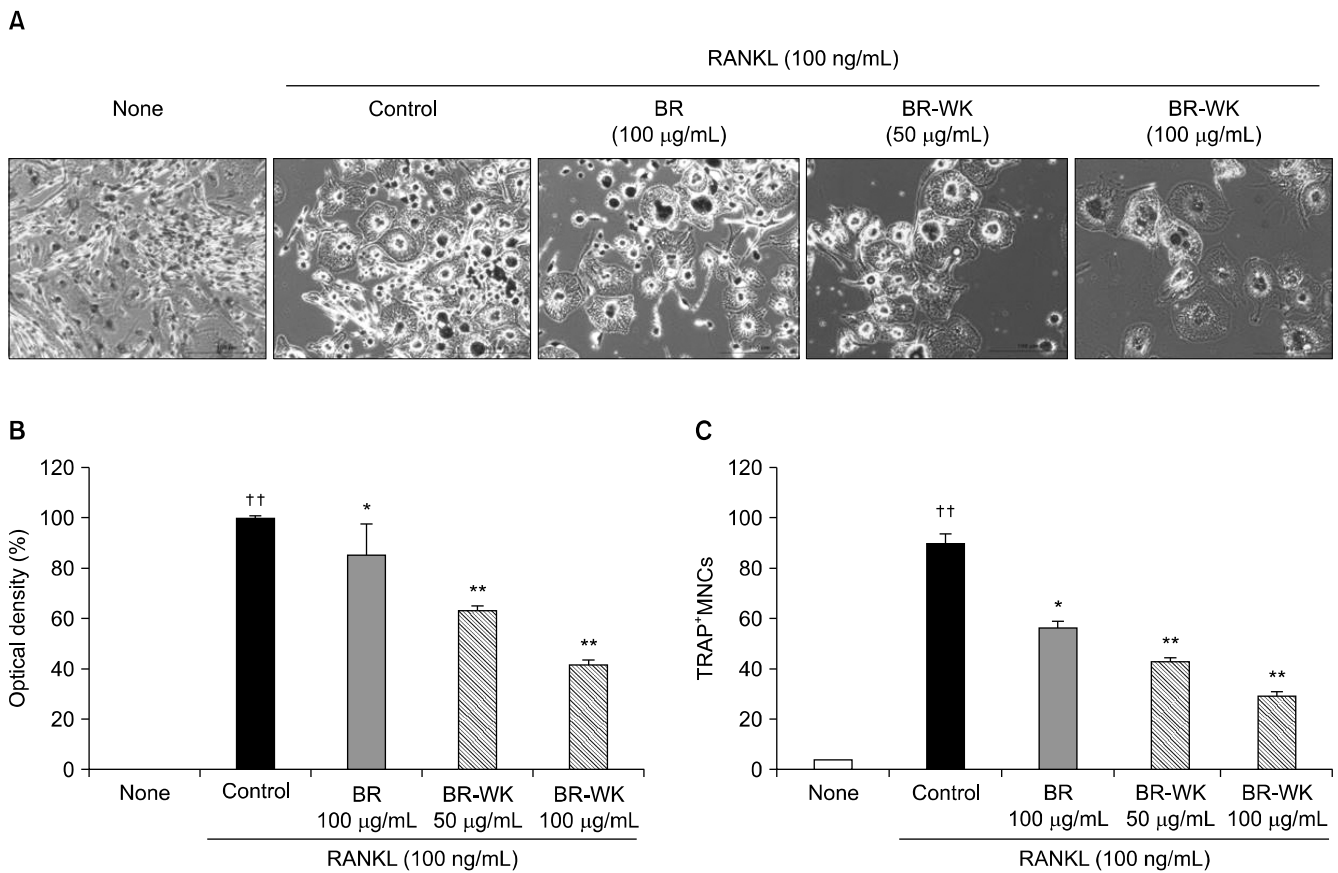
Fig. 3. BMMs cells were cultured with the indicated concentration of brown rice extract (BR) and brown rice extract treated by *Lactobacillus sakei* Wikim001 (BR-WK) in the presence of receptor activator of nuclear factor-κB ligand (RANKL, 100 ng/mL). After 5 days, the cells were fixed and stained for TRAP. BR-WK inhibited RANKL-induced osteoclast differentiation. (A) BR-WK inhibited RANKL-induced osteoclast differentiation in a dose-dependent manner. (B) BR-WK inhibited RANKL-induced osteoclast differentiation by absorption in a dose-dependent manner (405 nm). (C) BR-WK dose-dependently inhibited RANKL-induced formation of TRAP positive-multinucleated osteoclasts (TRAP+MNCs). The TRAP+MNCs were counted. Each experiment was performed in triplicate, and the results were expressed as the means±SD. ††$P<0.01$ vs. none; *$P<0.05$ vs. control; **$P<0.01$ vs. control.

extract and *L. sakei* Wikim001-treated brown rice extract on SaOS-2 cell proliferation were determined by the MTT assay. Fig. 1 illustrates the absorbance of formazan produced by cells on the BR-WK with and without being treated after 4 days in the culture. Compared to the control, higher absorbance was obtained when the SaOS-2 cells were treated with BR-WK.

The ALP activity was measured to observe the effects of BR-WK extract on osteoblast differentiation as an early osteoblastic marker. SaOS-2 cells were cultured with 100 μg/mL of BR, and 50 μg/mL or 100 μg/mL of BR-WK. BR-WK enhanced the ALP activity of the SaOS-2 cells in a similar way as observed with cell proliferation. In particular, the ALP activity significantly increased at BR-WK concentrations of 50 μg/mL and 100 μg/mL (Fig. 2). The ALP activities in the SaOS-2 cells were 130% higher when BR-WK was processed at a concentration of 100 μg/mL. Soy extract rich in isoflavones, such as genistein and daidzein, had a direct stimulatory effect on bone formation in cultured osteoblastic cells *in vitro*. The formation of MTT formazan was significantly increased up to 117% of the basal value when cells were treated with soy extract (14). BR-WK that showed 130% cell proliferation in the SaOS-2 cells is expected to have an effect on osteoporosis prevention.

Osteoporosis occurs when the osteoclast activity surpasses the osteoblast activity (20). Osteoclasts are large multinucleated cells of hematopoietic origin formed by differentiation and fusion of mononuclear monocyte-macrophage lineage precursors after stimulation with RANKL and M-CSF (21,22). The effect of BR-WK on osteoclast formation using bone marrow-derived macrophages was tested. 100 ng/mL of RANKL induced a TRAP positive multinucleated osteoclast differentiation in the BMMs cells. However, BR-WK inhibited osteoclast differentiation in a concentration-dependent manner (Fig. 3A). BR-WK reduced the number of TRAP-positive multinucleated cells generated with 37% and 59% inhibition at 50 μg/mL and 100 μg/mL concentrations, respectively (Fig. 3B). This culture system efficiently generated TRAP-positive multinucleated osteoclasts (Fig. 3A). When BR-WK was added to this culture-based osteoclastogenesis model, BR-WK dose-dependently decreased the numbers of osteoclasts generated (Fig. 3B and 3C). BR-WK demonstrated an almost 60% inhibitory effect at 100 μg/mL (Fig. 3C).

In conclusion, BR-WK stimulated the proliferation and activity of bone-forming osteoblasts while inhibiting the generation and activity of bone-resorbing osteoclasts. Although the active substances have not yet been identified, it is strongly suggested that BR-WK contains effective substances that have the potential to enhance bone metabolism in osteoporosis. *L. sakei* Wikim001 has high phytase activity and is beneficial in reducing phytate

contents in brown rice. By decreasing or eliminating its antinutritional effect, phytase also improves the bioavailability of nutritional factors. Improved bioavailability of brown rice is considered an effective method for the prevention of osteoporosis. These results indicate that BR-WK stimulates bone formation through its positive action on osteoblast differentiation and function and furthermore, decreases osteoclast differentiation.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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