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Original Article

The effect of Korean red ginseng on mesenchymal stem cells from healthy and osteoporotic human bone marrow

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Abstract: Background: Osteoporosis is a disease characterized by an increase in bone fragility as a result of decreased bone mass and weakening of the bone structure. There are studies on the relationship between osteoporosis and hearing and balance system. The goal of this study was to compare the proliferation and osteogenesis induction properties of mesenchymal stem cells derived from healthy and osteoporotic individuals to better understand the healing properties of Korean red ginseng (KRG) and Panax ginseng-Avena Sativa-Tribulus Terrestris mixture (PAT). Materials and methods: Osteoporotic and healthy MSCs were isolated successfully in culture conditions. The proliferation levels of cells treated with different doses of KRG and PAT were compared by Water-Soluble Tetrazolium-1 (WST-1) assay. Alkaline phosphatase (ALP) assay was performed by selecting the most effective KRG dose in proliferation. Results: Morphology of isolated cells and the expression of cell surface antigens have been detected as similar. The WST-1 assay showed that KRG was effective on the proliferation of osteoporotic cells. The levels of ALP in osteoporotic cells treated with KRG is increased depending on the differentiation day compared to healthy cells. Conclusion: KRG triggered an increase in intracellular ALP levels of osteoporotic MSCs. It suggests that KRG on osteoporotic cells is influential in stimulating osteogenesis and may be useful in osteoporotic patients.

Keywords: Korean red ginseng, mesenchymal stem cells, osteoporotic human bone marrow

Introductıon

Panax ginseng is a plant that grows in Asia and called Korean red ginseng (KRG) after heat treatment to increase its biological activities [1]. It means “full healing” in Greek. KRG has been widely used throughout the world for healing since old times and associated with antioxidant, anticancer and antidiabetic activities [2, 3]. *In vivo* studies to restore bone loss in osteoporotic mouse models and *in vitro* studies to determine the protective effects against apoptosis have been performed with Panax ginseng [1-4]. Avena sativa or so-called oats, which is rich in protein has been traded as grain for a long time. It has many bioactive components and has been used for medicinal purposes to help balance the menstrual cycle and to treat dysmenorrhoea, osteoporosis, and urinary tract infections [5]. Tribulus Terrestris is an extract from a plant commonly used in the Indian and Chinese traditional systems of medicine for the improvement of general health and helps to synthesize proteins and provides a positive nitrogen balance. The leaves and roots of this plant are used for different medical purposes. Tribulus Terrestris is also popular as an aphrodisiac [6]. In this study, we used PAT containing 22,4% Panax ginseng, 16,8% Avena sativa, 44,8% Tribulus Terrestris, 16% emulsifier and capsule which we used as a drug with KRG.

Osteoporosis is the most common form of the metabolic bone disease which is characterized

by a decrease in bone mineral and matrix according to the normal value, therefore, bone fragility is increased [7-9]. Especially in women and after menopause, the incidence is increasing [10].

On the other hand, the studies investigating the relationship between otosclerosis and audiological profile [11, 12] benign paroxysmal positional [13, 14] otosclerosis [15] are found in the literature. It has been a health problem that needs to be emphasized especially for societies with an extending life span.

Bone marrow contains cells which have differentiating ability to osteoblasts, chondrocytes, adipocytes and referred to as mesenchymal stem cells (MSCs) [7, 16]. Techniques for the isolation, culture, and identification of these cells with various markers have been developed. The isolation and culture of bone marrow (BM)-MSCs relies mainly on their ability to adhere to the bottom of the cell culture flasks. Isolated BM-MSCs have been shown to express specific cell surface markers that CD105 (transforming growth factor b receptor III), CD90 (thy-1), CD44 (hyaluronan receptor), CD73 (ecto 5 nucleotidase), CD146 (melanoma-cell adhesion molecule) and their expression of the hemopoietic markers including CD11b, CD45 and CD34 are missing [10, 17, 18].

In this study, we used osteoporotic human bone marrow-derived stem cells while using healthy human bone marrow-derived stem cells as a control group though bone marrow-derived stem cell studies are usually performed with healthy control groups. We aimed to determine the isolation, immunophenotypic properties of these cells and then the viability effects of KRG and PAT on these cells and Alkaline phosphatase levels with differentiation day. The influential effects in proliferation and stimulating osteogenesis were determined of KRG on osteoporotic human bone marrow mesenchymal stem cells.

Methods

Isolation, the culture of human bone marrow mesenchymal stem cells

Human bone marrow was used to isolate mesenchymal stem cells in project scope. 1-2 mL human BM (hBM) samples were obtained from healthy and osteoporotic individuals. 1-2 ml extra bone marrow was taken from the individuals who came to the clinic for diagnosis and after the diagnosis, healthy bone marrow samples were used as a control group.

The ethical declaration was obtained by KOU KAEK 20141106 research protocol code.

Bone marrow samples delivered to the laboratory were diluted 1:1 with PBS and centrifuged after spreading to the ficoll (Capricorn Scientific, Germany). After centrifugation, the collected buffy coat was washed with PBS and centrifuged again. Basal DMEM-F12 medium was added to the pellet and the cells were filtered with a 70-micron cell strainer. Cells were washed by centrifugation and were cultivated in T25 culture flasks in a medium composed of DMEM-F12 medium (Gibco, USA), 10% FBS (Gibco, USA), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Gibco, USA) in a 5% CO_2 atmosphere at 37°C.

Flow cytometry analysis

Flow cytometry analysis of isolated cells was performed to assess the immune profile of MSCs. Cells were collected from culture dishes and resuspended in PBS after centrifugation. For each sample 3×10^5 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD90, CD105, CD44, CD73, and CD45/34/11b/HLA DR/19 as a negative marker cocktail (ABclonal, USA) for 30 min at room temperature and the flow-cytometry analysis was performed using FACS-Calibur (BD Biosciences, Franklin Lakes, NJ, USA). The results were evaluated using the BD CellQuestTM software program.

WST-1 cell proliferation assay

KRG and PAT were weighed and dissolved in DMEM-F12 medium to prepare main stocks at a concentration of 2000 µg/ml. hBM-MSCs on passage 3 were seeded in a 96-well plate to be 1×10^4 cells in each well after trypsinization. KRG and PAT were diluted to concentrations of 15, 250, 1000 µg/ml and added to the medium as 2 replicates for each concentration. For day 1, 2 and 3, three plates were cultivated in the same condition, and 10 µl of Cell Proliferation Reagent WST-1 (Sigma-Aldrich, USA) was added to each plate after 1, 2 and 3 days. The plates exposed to the drug for 1, 2 and 3 days were incubated for 4 hours in incubator with WST-1

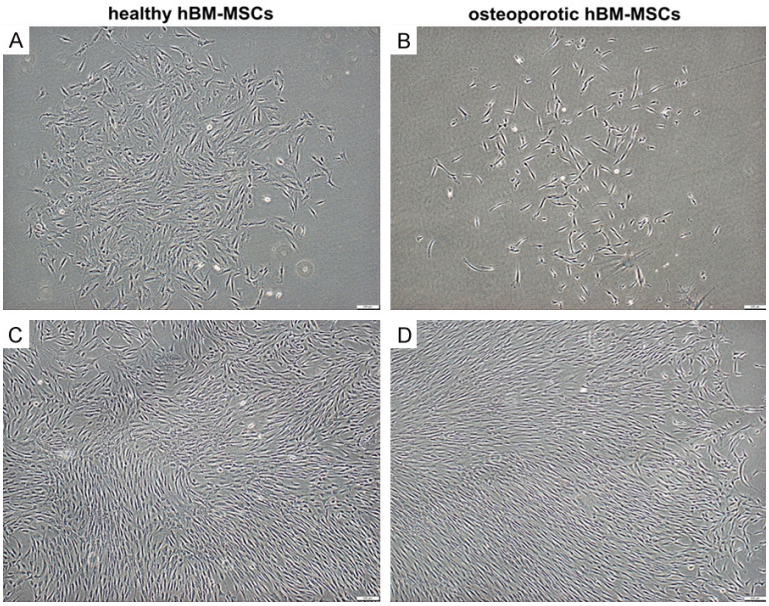

Figure 1. MSCs isolated from human bone marrow tissue were visualized by phase-contrast microscopy on day 6 (A, B) passage 0 (P0) and day 10 (C, D) P0 in adherent culture (Bars: 200 μm).

reagent and measured the absorbance of the samples at 480 nm. Charts were created according to OD values and the results were evaluated.

Alkaline phosphatase assay

Healthy and osteoporotic hBM-MSCs were seeded for osteogenic differentiation in 6-well plate to be 3×10^5 cells in each well for control, day 1, day 4, day 7, day 14 and day 21. 1000 μL/ml KRG was added to the differentiation medium and the differentiation medium was changed every 3 days. According to days, the cells were collected with a cell scraper from Petri dishes and precipitated in Eppendorf by centrifugation. The pellets were vortexed by adding the assay buffer from Alkaline Phosphatase Activity Colorimetric Assay Kit (BioVision, USA). After centrifugation, the supernatant was taken and protein samples were stored at -80°C. After the last 21 day cells were taken, the BCA Assay (Sigma-Aldrich, USA) was performed to quantitate all isolated proteins. Protein samples and standards were added with 2 replicates in 96-well plate. Mix solution formed with QA, QB, and Copper (II) sulfate were added to all the proteins and the plate was incubated for 1 hour at 60°C in dark. After incubation, the absorbance was measured at 562 nm and values were recorded.

For the ALP assay 0, 4, 8, 12, 16, 20 μl of 1 mM pNPP solutions were used to create standards and 20 μl of any protein sample was used to create background control and 20 μl of each of our samples were added as a duplicate in 96-well plate. Assay buffer was added to all samples and ALP activity was stopped by adding stop solution to the background controls. ALP enzyme was added to the standards and rapidly 5 mM pNPP solution was added to the samples and background controls. The plate was incubated in dark at 25°C for 60 minutes and after incubation stop solution was added to all other wells except the background controls. The absorbance at 405 nm was measured in a microplate reader. To calculate ALP activation, the individual ALP values of all samples were divided by the protein values and graphs were created with the calculated values.

Results

Morphology of hBM-MSCs

MSCs isolation was successfully performed from bone marrow aspirates of healthy donors and osteoporosis patients and morphology was examined every day by phase-contrast microscopy. From the earliest days of culture, the cells were observed as spindle-shaped. In the following days, it was observed that the cells were multiplying by forming colonies (**Figure 1**).

Flow cytometry analysis of hBM-MSCs

Flow cytometry analysis were performed with the immune profile of MSCs, and the isolated cells have positive expressions (**Figure 2**).

Characterization studies have shown that the isolated cells have characteristic features of hBM-MSCs.

Effects of KRG and PAT on hBM-MSCs viability

In this study, we performed a WST-1 assay to compare proliferation levels by treating both

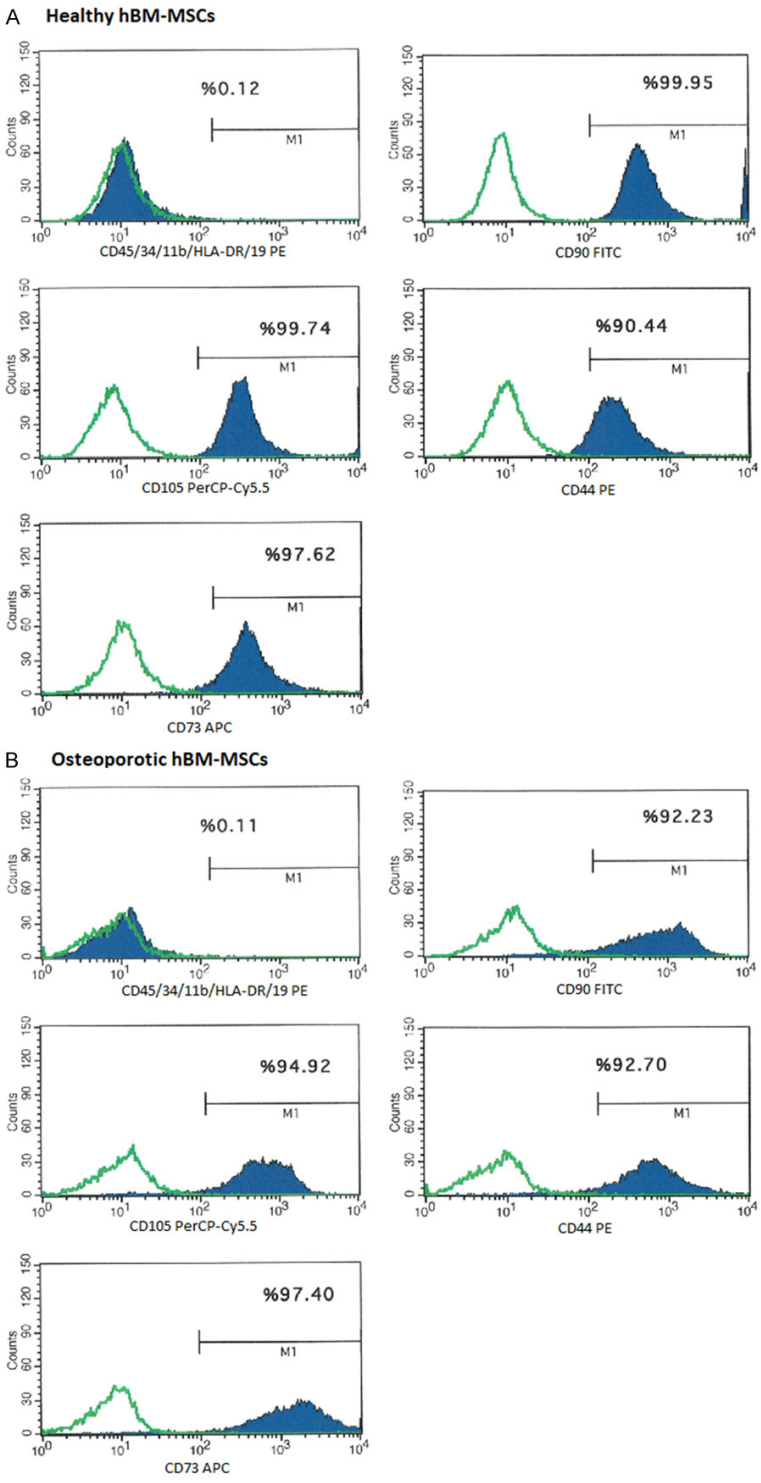

Figure 2. Flow cytometry analysis of hBM derived MSCs with the immune profile of MSCs (CD45/34/11b/HLA DR/19, CD90, CD105, CD44, CD73). Flow cytometry analysis of collected cells from culture dishes were performed. Positive expressions of CD90, CD105, CD44, CD73 and negative expressions of CD45/34/11b/HLA DR/19 marker cocktail indicate that isolated and analyzed cells have MSCs characteristic properties.

cells with KRG and PAT until day 3. There was no significant effect of both drugs on the proliferation of both cells detected on day 1.

Firstly, when comparing the effect of KRG on the proliferation of osteoporotic cells with dose increase, there is a regular and significant increase on the 3rd day when there is an irregular increase on the 2nd day. In particular, 1000 µg/ml KRG significantly increased the proliferation of osteoporotic cells. Along with that similar effect is seen for healthy cells, but this increase is not as high as for osteoporotic cells.

However, PAT did not cause a regular increase in the proliferation of cells. The proliferation of cells was increased on days 2 and 3 up to 250 µg/ml of dose and then decreased. In healthy cells, there was no clear effect after the 2nd day with a dose increase of PAT.

As a result, according to PAT, KRG showed a more prominent increase on the proliferation of cells with dose escalation, which was found to be significantly higher and more pronounced in osteoporotic cells than in healthy cells (**Figure 3**).

Alkaline phosphatase levels of hBM-MSCs

It is known that in osteoporosis the osteoblastic differentiation of MSCs is impaired and function of osteoblasts is decreased. Accordingly, ALP levels are also decreased. In this study, we attempted to improve this disorder by the addition of KRG.

According to WST-1 cell proliferation assay result, PAT showed no such increase, while KRG showed a more evident increase in proliferation of cells with the dose increase.

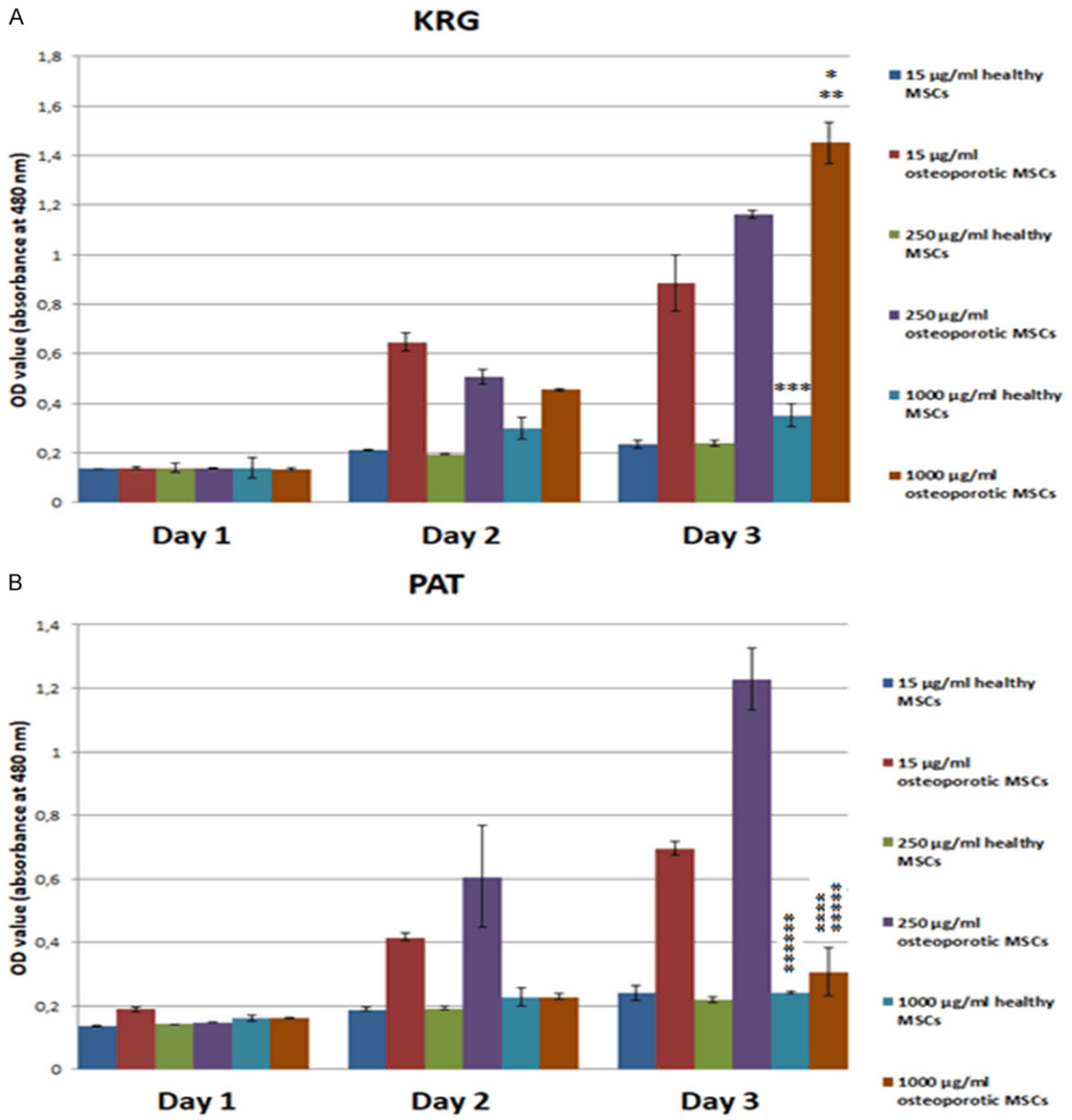

Figure 3. Evalution with column chart of WST-1 assay results with healthy and osteoporotic hBM derived MSCs depending on time (day 1, 2, 3) and concentration of KRG and PAT (15, 250, 1000 µg/ml). (*P<0.001 versus healthy MSCs with 1000 µg/ml KRG on day 3. **P<0.01 versus osteoporotic MSCs with 1000 µg/ml KRG on day 1. ***P<0.05 versus healthy MSCs with 1000 µg/ml KRG on day 1. ****P>0.10 versus healthy MSCs with 1000 µg/ml PAT on day 3. *****P<0.10 versus osteoporotic MSCs with 1000 µg/ml PAT on day 1. ******P<0.01 versus healthy MSCs with 1000 µg/ml PAT on day 1) (KRG: Korean red ginseng, PAT: 22,4% Panax ginseng, 16,8% Avena sativa, 44,8% Tribulus Terrestris, 16% emulsifier and capsule.

Therefore the ALP Assay was performed with 1000 µg/ml KRG dose as the most appropriate dose for cell proliferation.

Osteogenic differentiation of MSCs was quantified by monitoring ALP activity (**Figure 4**). Intracellular ALP was found to peak and there was greater calcium deposition when osteoporotic hBM-MSCs were incubated with KRG rather than healthy IBM-MSCs. While the amount of ALP does not increase highly in healthy hBM-MSCs with differentiation day, there was a regular increase in osteoporotic hBM-MSCs from day 1 with differentiation day, and markedly increased on day 21. As a result, it has been suggesting that KRG on osteoporotic cells is influential in proliferation and stimulating osteogenesis.

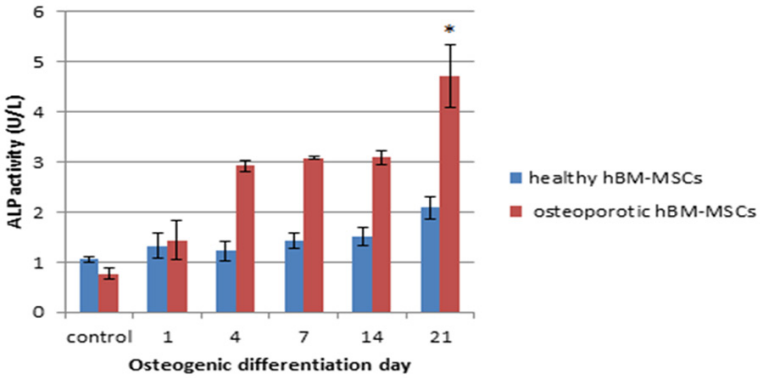

Figure 4. Effect of 1000 μg/ml KRG on ALP activity of hBM-MSCs. Cells were treated with 1000 μg/ml KRG and exposed to osteogenic differentiation medium for 1, 4, 7, 14, 21 days and exposed to normal DMEM-F12 medium as a control group. ALP activity was analyzed and calculated by comparison with total protein levels. *P<0.01 versus osteoporotic control (KRG: Korean red ginseng).

As a result of the WST-1 assay, the proliferation of osteoporotic cells increased regularly by dose and day increase, especially on day 3. However, KRG is ineffective in healthy cells, and there is no significant increase in proliferation with the dose increase. Furthermore in both PAT applied cells from healthy and osteoporotic individuals, there was no regular increase in proliferation by dose and day increase. PAT has no significant effect on the proliferation of both cells.

Alkaline phosphatase assay was performed with KRG from the conclusion that the effect of KRG is more evident than PAT. Additionally, 1000 μg/ml KRG dose has been selected for ALP assay as the most appropriate dose for cell proliferation. ALP levels of healthy and osteoporotic cells were compared with differentiation days. However there was only a slight increase in healthy cells with differentiation days, increases in osteoporotic cells were more pronounced and intracellular ALP levels were significantly increased on day 21 of differentiation. These results showed that KRG can induce osteogenesis with calcium deposition in MSCs derived from osteoporotic patients. Therefore, it is concluded that the use of KRG in patients stricken from osteoporosis due to menopause or other reasons may be useful however clinical trials are needed.

Discussion

MSCs derived from osteoporotic and healthy donors might be isolated from bone marrow and expanded in culture [16, 17]. These isolated stem cells have similar morphology and express similar cell surface antigens as evidenced by their reactivity with cell-specific monoclonal antibodies [17]. However, it has been shown that MSCs from osteoporotic patients have a lower rate of proliferation than healthy cells under *in vitro* conditions [19].

It has been shown in previous studies that KRG inhibits apoptosis of cells, increases expression of osteogenic genes (ALP, OCN, OPN, Runx2, and BMPs) *in vitro* and increases bone formation in osteoporotic mice *in vivo* [1, 20]. Ginseng has also been shown to inhibit bone loss by inhibiting osteoclast differentiation, a process controlled by estrogen [21]. Additionally, there are many studies have shown that ginseng can reduce osteoporosis by stimulating ALP, Col-I, Runx2, inhibiting NF-kB, and increasing blood circulation [3].

In this study, we aimed to demonstrate the possible effects of KRG and PAT on proliferation and *in-vitro* osteogenic differentiation processes on MSCs from healthy and osteoporotic individuals. For this purpose, we performed WST-1 cell proliferation assay and ALP assay by applying KRG and PAT to healthy and osteoporotic MSCs.

Conclusion

MSCs derived from osteoporotic patients have significant differences in response to KRG treatment compared to control donors. This result suggests that KRG may be a new approach to the treatment of osteoporosis. Further studies are needed to elucidate the molecular mechanisms that explained the results of the increase in proliferation and calcium deposition of cells in this study. Illumination of this mechanism may lead to the development of new therapeutic strategies for the treatment of osteoporosis.

Disclosure of conflict of interest

None.

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