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# *Cuscuta chinensis* Extract Promotes Osteoblast Differentiation and Mineralization in Human Osteoblast-Like MG-63 Cells

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ABSTRACT The aim of the present study was to investigate whether the aqueous extract of *To-Sa-Za* (TSZ-AE), the seed of *Cuscuta chinensis* Lam., which is a traditional medicinal herb commonly used in Korea and other oriental countries, could induce osteogenic activity in human osteoblast-like MG-63 cells. TSZ-AE treatment mildly promoted the proliferation of MG-63 cells at doses of 500 and 1,000 $\mu$g/mL in the 24-hour culture period. Dose-dependent increases in alkaline phosphatase (ALP) activity and collagen synthesis were shown at 48 and 72 hours of incubation. The release of bone morphogenetic protein (BMP)-2 but not osteocalcin in the MG-63 cells was induced by TSZ-AE at 72 hours (100–1,000 $\mu$g/mL). In addition, TSZ-AE markedly increased mRNA expression of ALP, collagen, and BMP-2 in the MG-63 cells in a dose-dependent manner. Mineralization in the culture of MG-63 cells was significantly induced at 500 and 1,000 $\mu$g/mL TSZ-AE treatment. In conclusion, this study shows that TSZ-AE enhanced ALP activity, collagen synthesis, BMP-2 expression, and mineralization in MG-63 cells. These results strongly suggest that *C. chinensis* can play an important role in osteoblastic bone formation and may possibly lead to the development of bone-forming drugs.

**KEY WORDS:** • ***alkaline phosphatase*** • ***mineralization*** • ***osteoblast differentiation*** • ***osteocalcin***

## INTRODUCTION

OSTEOBLASTS ARE BONE-FORMING cells that originate from mesenchymal progenitors. With appropriate stimulation, such as bone morphogenetic proteins (BMPs) and transforming growth factor (TGF),[1,2] mesenchymal progenitors undergo proliferation and differentiate into preosteoblasts and then mature into functional osteoblasts.[3] In culture, as *in vivo*, osteoblasts form bone-like mineralized nodules by undergoing three stages of development: proliferation, extracellular matrix maturation, and mineralization.[4,5] During each stage of development, specific subsets of genes are sequentially expressed or repressed. For example, histone 4 is a marker for proliferation, alkaline phosphatase (ALP) for extracellular matrix maturation, and osteocalcin for mineralization.[5]

BMPs are growth/differentiation factors of the TGF superfamily that were originally defined by their ability to induce formation of both cartilage and bone when implanted at ectopic sites.[6] Among BMPs, BMP-2 regulates osteoblast differentiation *in vitro*[7,8] and can also stimulate bone resorption by directly stimulating the differentiation of osteoblasts and activating mature osteoclasts.[9,10] It has been shown that BMP-2 is capable of driving the murine pre-myoblast cell line C2C12 cells into osteoblastic lineage by suppressing the expression of myogenic genes and inducing that of osteogenic genes.[11]

Type I collagen accounts for over 90% of the organic matrix of bone.[12] Previous investigations demonstrated that type I collagen regulate the differentiation of osteoblasts. Indeed, a collagen-precoated dish or collagen gel increases the activity of osteoblasts and promotes the osteogenic differentiation of mesenchymal progenitors cells.[13] In addition, the interaction of type I collagen with the cell surface $\alpha 2\beta 1$ integrin receptor of mouse osteoblasts is required for the osteoblastic differentiation,[14,15] and type I collagen–$\alpha 2\beta 1$ integrin interaction is an important signal for the osteoblastic differentiation of rat mesenchymal progenitors cells.[16]

Osteoporosis is a condition characterized by low bone mass and structural deterioration of the bone tissue, leading to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist.[17] Its etiology may be related to a number of factors, including dietary status, hormone deficiency, lack of weight-bearing activity, and abnormal regulation of calcium homeostasis.[18] Postmenopausal osteoporosis (type I osteoporosis) is linked to ovarian hormone deficiency and is characterized by an increased rate of bone turnover, with resorption exceeding formation.[19] Secondary or type II osteoporosis

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is related to factors other than estrogen, such as dietary disorders, lifestyle, sex hormone deficiency and other endocrine disorders, bone and bone marrow disease, kidney and liver disease, and long-term drug treatment with corticosteroids.[20,21] Recently, there has been increasing demand for herbal medicines that are deemed to be healthier and safer for the treatment of osteoporosis. Indeed, Chen *et al*.[22] demonstrated that icariin, a prenylated flavonol glycoside contained in the herb *Epimedium*, enhances the osteogenic differentiation of rat bone marrow stromal cells.

*To-Sa-Za* is the dry seed of *Cuscuta chinensis* Lam. (Family Convolvulaceae), which is collected in the fall when the seed is mature. It is an important herbal medicine that is widely used as a tonic and aphrodisiac to nourish the liver and kidneys and to treat impotence and seminal emission.[23–28] Moreover, it is considered to have an antitumor effect in prostate and skin carcinomas.[25,26] Recent studies have demonstrated that *C. chinensis* has a neuroprotective effect on neurons from ischemic damage and neuronal cell toxicity[27] and enhances memory by inducing PC12 cell differentiation.[28] Although several studies have demonstrated the biological functions of *C. chinensis*, they were limited on neuroprotective and antitumor effects.[23–28] Yao *et al*.[29] demonstrated that addition of an aqueous extract of *C. chinensis* in rat bone cells clearly promoted the proliferation and differentiation of the osteoblasts, but inhibited the activities of osteoclasts. These observations suggested that *C. chinensis* has osteogenic effects. However, the osteogenic effects of the seeds of *C. chinensis* (*To-Sa-Za*) are unknown. Therefore, the present study was performed to clarify the effect of the aqueous extract of *To-Sa-Za* (TSZ-AE) on the proliferation, differentiation, and mineralization of osteoblasts in human osteoblast-like MG-63 cells.

## MATERIALS AND METHODS

### *Reagents*

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium, penicillin, streptomycin, and fetal bovine serum were purchased from Hyclone (Logan, UT). CellTiter 96® $AQ_{ueous}$ One Solution and reverse transcription system were purchased from Promega (Madison, WI). A SYBR® green polymerase chain reaction (PCR) kit was purchased from Qiagen (Hilden, Germany). BMP-2 and osteocalcin enzyme-linked immunosorbent assay (ELISA) kits were manufactured by R&D Systems (Minneapolis, MI) and Takara (Kyoto, Japan), respectively. The BCA™ protein assay kit was purchased from Pierce (Rockford, IL). TRIzol reagent was obtained from Invitrogen (Grand Island, NY). The antibodies against Smad and phospo-Smad-1/5/8 were obtained from Cell Signaling Technology (Danvers, MA).

### *Preparation of TSZ-AE*

The seeds of *C. chinensis* Lam. were purchased from Jeil Oriental Medicine Clinic (Seoul, Republic of Korea). For extraction, 1.5 kg of *C. chinensis* Lam. was ground and extracted with boiling water for 4 hours in a water extractor (Kyungseo Machines, Inchon, Republic of Korea). After 30 minutes of centrifugation at 3,000 *g*, the supernatant was concentrated by rotary vacuum evaporation and then lyophilized with a freeze dryer (Ilshinlab, Yangju, Republic of Korea). The extract yield was 30% (wt/wt).

### *Cell culture and cell viability assay*

The human osteoblastic cell line, MG63, purchased from the Korean Cell Bank (Seoul), was cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 $\mu$g/mL streptomycin) and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. The effects of the TSZ-AE on cell viability were tested using the CellTiter 96 $AQ_{ueous}$ One Solution assay, which uses colorimetry to count the number of viable cells. This assay was used to determine the number of viable cells remaining after the culturing process was complete. MG-63 cells ($5 \times 10^3$) were seeded on 96-well plates and incubated with 100, 500, or 1,000 $\mu$g/mL TSZ-AE. After 24-, 48-, and 72-hour incubation periods, the number of viable cells was measured according to the manufacturer's instructions.

### *Analysis of ALP activity*

MG-63 cells ($1 \times 10^5$) were seeded on six-well plates and incubated with TSZ-AE at various concentrations. After 24-, 48-, and 72-hour incubation periods, the cell layer was washed with Dulbecco's phosphate-buffered saline (DPBS) and dissolved in 0.25% Triton X-100. The cell lysate was then sonicated for 30 seconds on ice. The cellular protein concentration was determined after incubation in a protein assay reagent, using the BCA protein assay kit. The ALP activity was measured using a spectrophotometric method with *p*-nitrophenyl phosphate as the substrate. The optical density was measured at 405 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA). The results, which were normalized to protein concentration, are expressed as a percentage of the control.

### *Determination of collagen content*

MG-63 cells ($1 \times 10^5$) were seeded on six-well plates and incubated with TSZ-AE at various concentrations. After 24-, 48-, and 72-hour incubation periods, the cells and cell culture supernatants were collected in order to determine collagen contents. The cells were lysed by addition of 0.1% Triton X-100 and centrifuged at 500 *g* for 5 minutes, and supernatants were tested for their collagen contents. The cell culture supernatants were centrifuged at 10,000 *g* for 3 minutes, and the supernatants were stored at −70°C until use. Collagen standards were prepared using collagen type I, and calibration was obtained for their absorbance values in a range of 0–400 $\mu$g/mL. After being thawed, the collected supernatants were centrifuged again. Fifty microliters of su-

pernatant, sample, and standard was added in each well of a 96-well plate. The plates containing the supernatant, sample, and standard were incubated at 37°C consecutively for 16 hours in a humidified atmosphere and 24 hours in a dry oven. After incubation, all the wells were washed three times with 200 $\mu$L of distilled water. Clean wells were filled with 100 $\mu$L of 0.1% (wt/vol) Sirius red F3BA in saturated picric acid. Staining lasted 1 hour at 37°C in a dry incubator. The plates were finally washed five times for 10 seconds with 200 $\mu$L of 10 m*M* HCl solution. The samples underwent a further step in 0.1 *N* NaOH for 5 minutes at room temperature to dissolve the adsorbed collagen. The solution was mixed several times and transferred into a clean plate. Finally, the absorbance at 540 nm was measured using an ELISA reader. Collagen synthesis was normalized to the protein concentration, and the protein concentration was assessed by the BCA protein assay kit.

### *Assaying levels of osteocalcin and BMP-2*

Osteocalcin and BMP-2 ELISA kits were used to detect the osteocalcin and BMP-2 levels, respectively. In brief, cells were treated with various concentrations of TSZ-AE for the indicated times. The amounts of osteocalcin and BMP-2 in the cell culture supernatants were measured using an ELISA kit.

### *Analysis of mineralization*

MG-63 cells ($2.5 \times 10^4$) were seeded on 12-well plates and incubated with TSZ-AE at various concentrations. The degree of mineralization was determined using Alizarin red S staining after 5- and 7-day treatment with TSZ-AE. In brief, the cells were fixed with ice-cold 70% (vol/vol) ethanol for 1 hour and then stained with 40 m*M* Alizarin red S in distilled water (pH 4.2) for 10 minutes at room temperature. After removal of the Alizarin red S solution by aspiration, the cells were incubated in DPBS for 15 minutes at room temperature on an orbital rotator and then rinsed once with fresh DPBS. The cells were subsequently destained for 15 minutes with 10% (wt/vol) cetylpyridinium chloride in 10 m*M* sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using an ELISA reader.

### *Real-time PCR for ALP, collagen, osteocalcin, and BMP-2*

Using Trizol reagent, total RNA was extracted from the MG63 cells that were cultured in the presence of various concentration of TSZ-AE for various periods in six-well plates. The total amount of harvested RNA was measured using an ultraviolet/visible spectrophotometer at 260 nm. One microgram of the total RNA was reverse-transcribed to cDNA using a reverse transcription system (Promega). Real-time PCR was carried out using the Rotor-gene 3000 PCR (Corbett Research, Sydney, Australia) and the SYBR green PCR kit. The primer sequences were as follows: ALP gene, upstream primer 5′-CCCAAAGGCTTCTTCTTG-3′ and downstream primer 5′-CTGGTAGTTGTTGTGAGCAT-3′[30]; osteocalcin gene, upstream primer 5′-ATGAGAGCC-CTCACACTCCTC-3′ and downstream primer 5′-GCCG-TAGAAGCGCCGATAGGC-3′[30]; BMP-2 gene, upstream primer 5′-GCAGGCACTCAGGTCAGC-3′ and downstream primer 5′-AGAAGAATCTCCGGGTTGTTT-3′[31]; type I collagen gene, upstream primer 5′-TGACCTCAA-GATGTGCCACT-3′ and downstream primer 5′-ACCA-GACATGCCTCTTGTCC-3′[32]; and $\beta$-actin gene, upstream primer 5′-TGACCCAGATCATGTTTGAGA-3′ and downstream primer 5′-ACTCCATGCCCAGGAAGGA-3′. The data obtained were normalized to $\beta$-actin.

### *Statistical analysis*

The data are presented as mean ± SEM values from at least three independent experiments. The values were evaluated by one-way analysis of variance, followed by Duncan's multiple range tests using Prism version 4.0 software (GraphPad Software Inc., San Diego, CA). Differences were considered significant at $P < .05$.

## RESULTS

### *Effect of TSZ-AE on viability in osteoblast-like MG-63 cells*

We first determined the effect of TSZ-AE on the viability of MG-63 cells. TSZ-AE mildly stimulated proliferation of the MG-63 cells at 500 and 1,000 $\mu$g/mL in the 24-hour culture period (Fig. 1). Since TSZ-AE did not show any negative effect on cell viability of MG-63 cells after 3 days at increasing doses, we used the same doses for the continuing experiments.

### *Effect of TSZ-AE on osteoblastic differentiation*

Since ALP activity is a major osteoblastic differentiation marker,[5] we examined the effect of TSZ-AE on osteoblastic differentiation by measuring ALP activity. As shown in

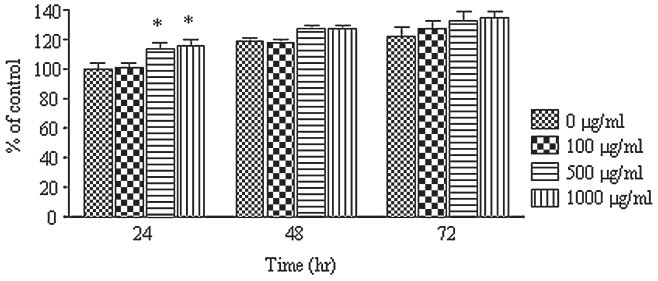


**FIG. 1.** Effects of TSZ-AE on viability in human osteoblast-like MG-63 cells. MG-63 cells were treated with 0, 100, 500, and 1,000 $\mu$g/mL TSZ-AE for 24, 48, and 72 hours. Cell viability was determined by a colorimetric CellTiter 96 $AQ_{ueous}$ One Solution assay. Data are mean ± SEM values from three experiments. *$P < .05$ compared with 0 $\mu$g/mL-treated MG-63 cells.

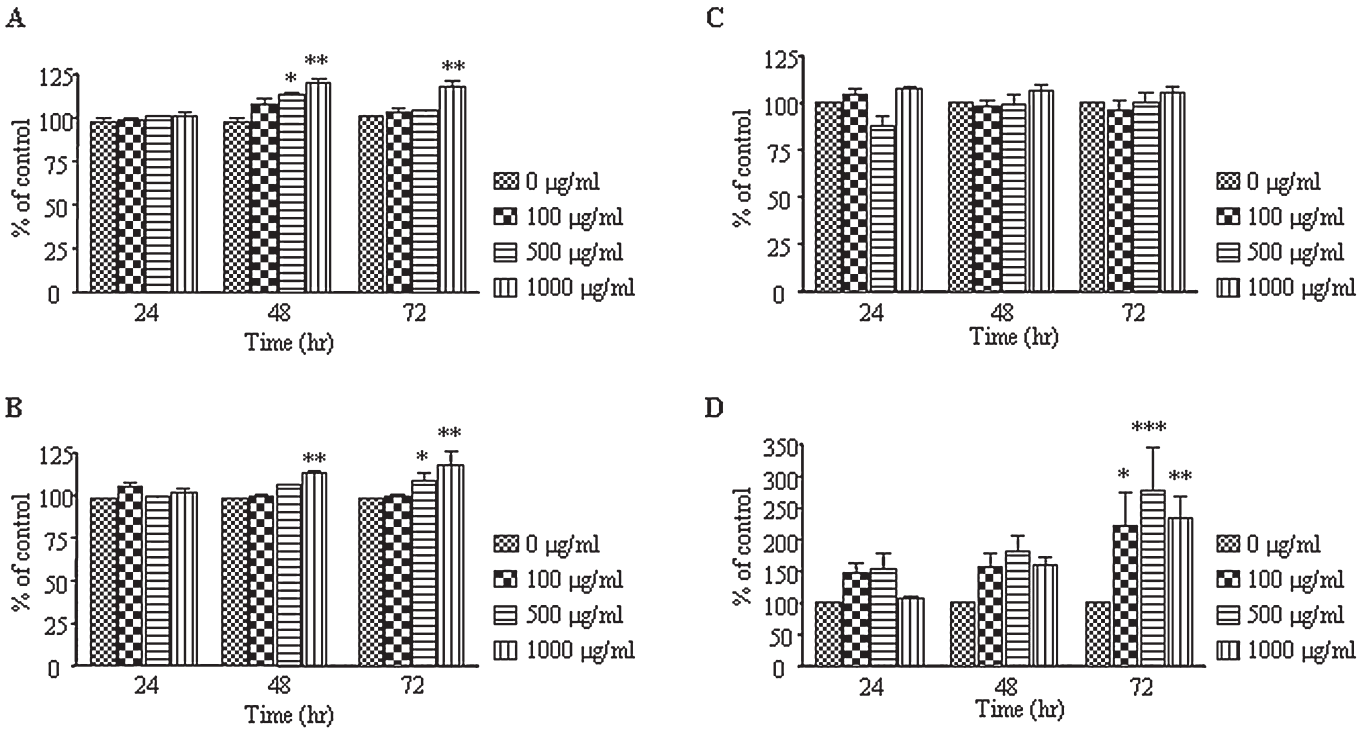


**FIG. 2.** Effects of TSZ-AE on (**A**) ALP activity, (**B**) collagen synthesis, and production of (**C**) osteocalcin and (**D**) BMP-2 in human osteoblast-like MG-63 cells. MG-63 cells were treated with 0, 100, 500, and 1,000 $\mu$g/mL TSZ-AE for 24, 48, and 72 hours. ALP activity, collagen synthesis, and osteocalcin and BMP-2 production were determined as described in Materials and Methods. Data are mean ± SEM values from three experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ compared with 0 $\mu$g/mL-treated MG-63 cells.

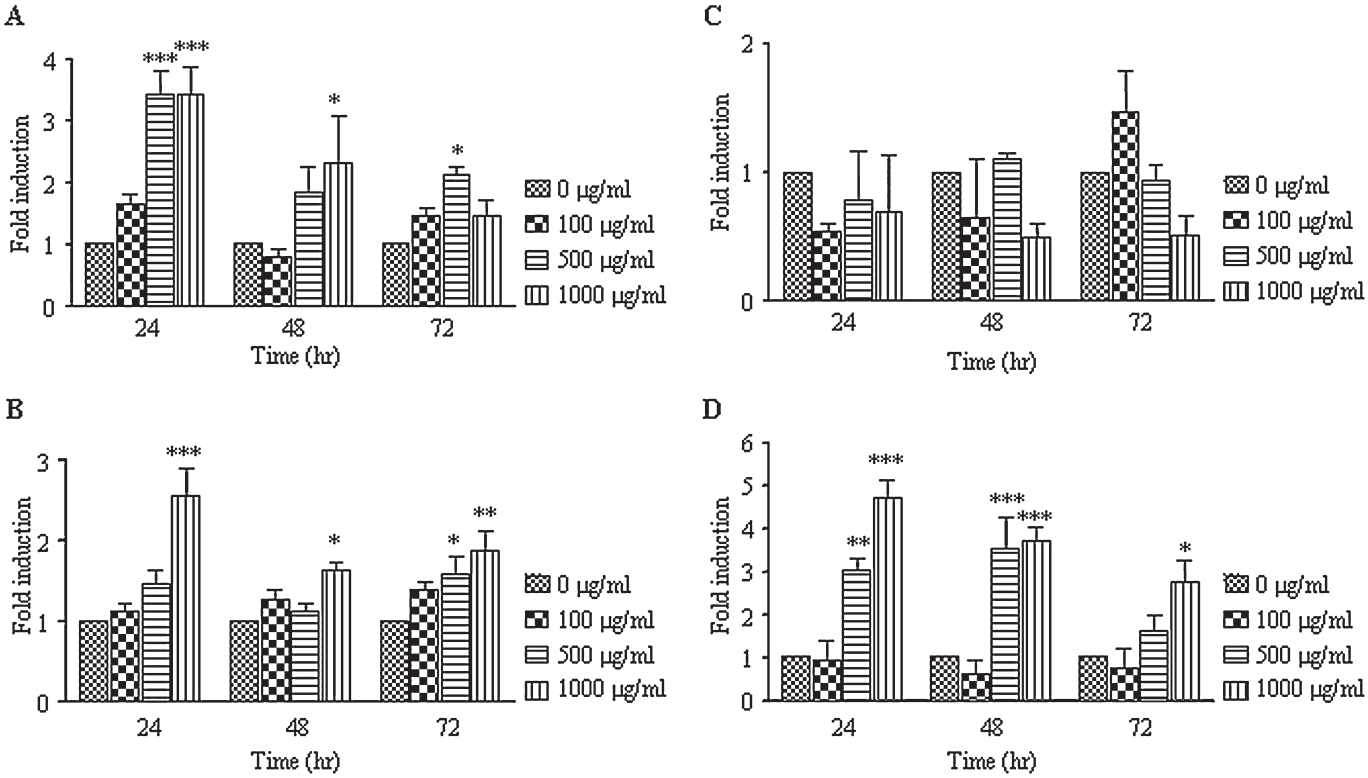


**FIG. 3.** Effects of TSZ-AE on mRNA expressions of (**A**) ALP, (**B**) collagen, (**C**) osteocalcin, and (**D**) BMP-2 in human osteoblast-like MG-63 cells. MG-63 cells were treated with 0, 100, 500, and 1,000 $\mu$g/mL TSZ-AE for 24, 48, and 72 hours. ALP, collagen, osteocalcin, and BMP-2 mRNA expressions were determined by real-time reverse transcription-PCR as described in Materials and Methods. Data are mean ± SEM values from three independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ as compared with 0 $\mu$g/mL-treated MG-63 cells.

Figure 2A, 24 hours of incubation with TSZ-AE did not alter the ALP activity of the MG-63 cells; however, significantly increased ALP activity was shown in the cells over the 2- and 3-day cultures at the concentration of 1,000 $\mu$g/mL (Fig. 2A).

Collagen content is another marker of osteoblastic differentiation, and collagen induces the activity of ALP.[5,33] Therefore, we examined the effect of TSZ-AE on collagen synthesis and ability. As shown in Figure 2B, the ability to synthesize collagen in the TSA-AE-exposed cells was similar to the effects on ALP activity. The collagen contents of the cells and cell culture supernatants were significantly increased by addition of TSZ-AE at the dose of 1,000 $\mu$g/mL as compared to the nontreated control group at days 2 and 3.

Next, we observed the effects of TSA-AE on production of osteocalcin and BMP-2 in MG-63 cells. The MG-63 cells were treated with various concentrations of TSZ-AE (0–1,000 $\mu$g/mL), and the contents of osteocalcin and BMP-2 in the medium were measured. There was no significant increase in osteocalcin release by treating the MG-63 cells with TSZ-AE (Fig. 2C). However, TSZ-AE treatment exhibited greatly elevated BMP-2 secretion in the osteoblastic cells. After 3 days of culture with 100, 500, and 1,000 $\mu$g/mL TSZ-AE, BMP-2 production in the MG-63 cells increased significantly 2.3-fold, 2.8-fold, and 2–5 fold, respectively, as compared to the nontreated MG-63 cells (Fig. 2D).

### *Effect of TSZ-AE on mRNA synthesis of ALP, collagen, osteocalcin, and BMP-2*

In order to further provide insight into the molecular mechanism underlying the osteogenic effects of TSZ-AE, mRNA expression levels of osteoblast differentiation markers were measured by real-time reverse transcription-PCR. The treatments with 500 and 1,000 $\mu$g/mL TSZ-AE for 24 hours induced the gene expression of ALP 3.3-fold above the baseline levels (Fig. 3A). Similar to collagen contents, mRNA expression of type I collagen was induced by 1,000 $\mu$g/mL TSZ-AE treatment with 24-, 48-, and 72-hour incubation periods (Fig. 3B). However, TSZ-AE treatment did not have a significant effect on osteocalcin mRNA expression (Fig. 3C), while BMP-2 mRNA expression was dramatically increased by addition of TSZ-AE as compared to the nontreated MG-63 cells (Fig. 3D). Treatment of the MG-63 cells with 500 and 1,000 $\mu$g/mL TSZ-AE for 24 hours resulted in threefold and fivefold inductions of BMP-2 expression, respectively (Fig. 3D). The induction of BMP-2 mRNA by TSZ-AE was shown up until the 72-hour incubation period.

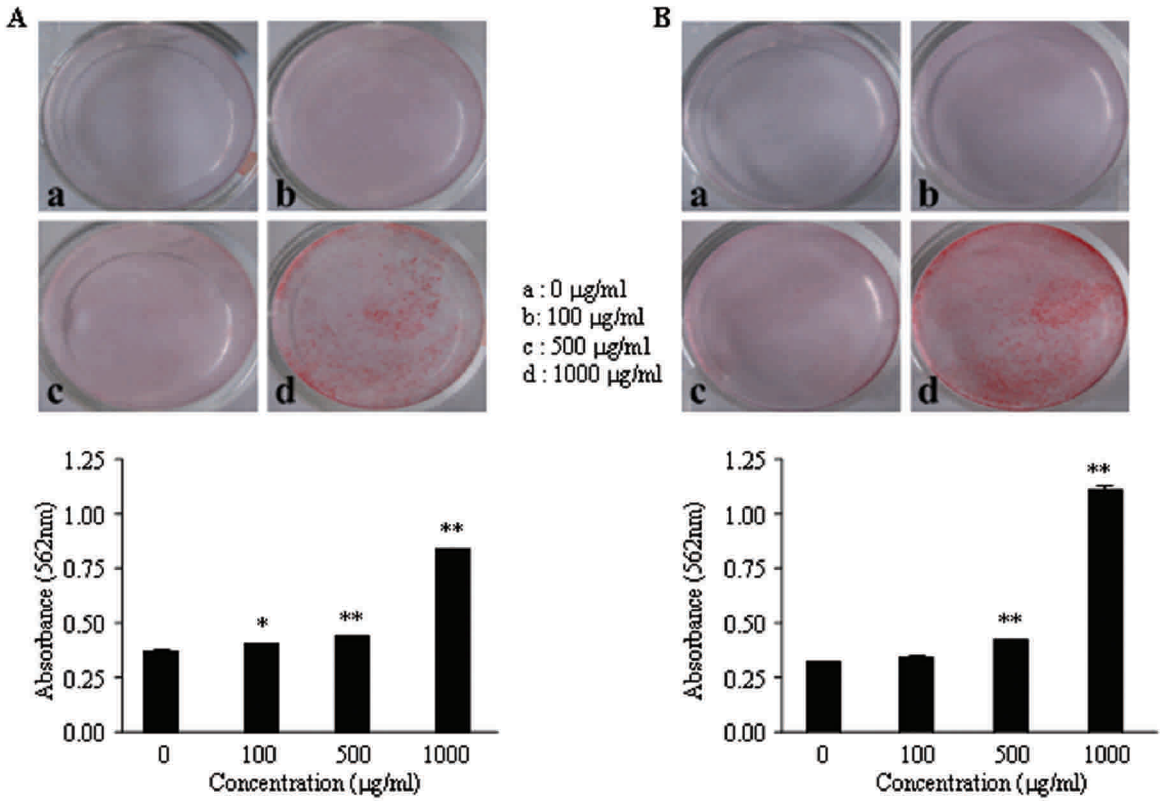


**FIG. 4.** Effects of TSZ-AE on mineralization in human osteoblast-like MG-63 cells. MG-63 cells treated with 0, 100, 500, and 1,000 $\mu$g/mL TSZ-AE for (**A**) 5 and (**B**) 7 days. Alizarin Red-S staining for calcium mineralization and mineral deposition in the MG-63 cells was determined as described in Materials and Methods. Data are mean ± SEM values from three independent experiments. $^*P < .05$, $^{**}P < .001$ as compared with 0 $\mu$g/mL-treated MG-63 cells.

*Effect of TSZ-AE on mineralization*

Finally, in order to examine the effect of TSZ-AE on mineralization in MG-63 cells, mineralization was detected with Alizarin red S staining. Alizarin red S staining is a standard method for visualizing nodular patterns and calcium deposition in MG-63 cell cultures *in vitro*. As shown in Figure 4A, both the 500 and 1,000 $\mu$g/mL-treated MG-63 cells showed a clearer red color than the nontreated group. The amount of mineralization significantly increased in the 500 and 1,000 $\mu$g/mL TSZ-AE-treated groups at 5 and 7 days (Fig. 4B).

## DISCUSSION

To investigate the effect of TSZ-AE on bone metabolism, we employed a cell culture system using MG-63 cells. MG-63 cells exhibit osteoblast-like characteristics such as the production of osteocalcin and type I collagen.[33,34] With their osteoblast-like phenotype, MG-63 cells serve as a useful model to test the biological performance of various materials.[35,36] First, we determined whether TSZ-AE inhibits the proliferation of osteoblasts. TSZ-AE had no negative effect on the proliferation of the MG-63 cells as shown in Figure 1. This result indicates that TSZ-AE is nontoxic to osteoblastic cells, which suggests the possibility for reducing side effects.

ALP activity is the most widely recognized biochemical marker for osteoblastic activity and is believed to play a role in bone mineralization.[3–5] Therefore, we examined the effect of TSZ-AE on the ALP activity of MG-63 cells. TSZ-AE increased both ALP activity and mRNA synthesis. These results indicate TSZ-AE stimulates the proliferation and differentiation of osteoblasts by enhancing the synthesis of ALP, at least in part (Fig. 2A).

In order to determine the response of the MG-63 cells to TSZ-AE in terms of extracellular matrix protein expression, we observed collagen synthesis and osteocalin expression. Osteocalin is a later marker of osteoblastic differentiation that is closely related to osteoblastic maturation.[33] In addition, osteoblasts abundantly synthesize and secrete type I collagen, a major bone matrix constituent and extracellular macromolecule in osteoblast cultures.[33,35] We did not observed inductions of osteocalcin mRNA and protein expression by TSZ-AE; however, the synthesis and secretion of type I collagen as well as mRNA expression were increased by TSZ-AE treatment in the MG-63 cells (Figs. 2B and 3B). Also, we found that TSZ-AE induced mineralized nodule formation at 5 and 7 days of MG-63 cell culture (Fig. 4). This result supports the hypothesis that TSZ-AE promotes osteoblastic differentiation *in vitro*, via the increased synthesis and secretion of matrix proteins.

BMPs, which belong to the TGF-$\beta$ superfamily, were originally identified as compounds that induce bone and cartilage formation.[6–8] Extensive studies have demonstrated that BMPs, including BMP-2, are potent bone cell-differentiating factors as well as bone formation stimulators.[6–9] Therefore, we determined the mRNA and protein levels of BMP-2 after TSZ-AE treatment. Our study indicates that BMP-2 production and mRNA expression increase in TSZ-AE-treated MG-63 cells. However, the phosphorylation of SMAD protein was not induced by TSA-AE treatment of the MG-63 cells (data not shown).

The main constituents of *C. chinensis* have been shown to be flavonoids, saccharide, alkaloids, lignans, and resin glycosides.[37–40] These compounds are suggested as being responsible for the pharmacologic activities observed from *C. chinensis*.[37,38] The exact component(s) responsible for the osteogenic effects may be found in the aqueous extracts of the dry seed of *C. chinensis* Lam., as the experimental evidence from our study suggests. Furthermore, such activity appears to be mediated through the ability of these compounds to induce the molecules involved in osteoblast differentiation and mineralization.

Among the studies of various biological effects of *C. chinensis*, there is no direct experimental evidence of the therapeutic benefits of *C. chinensis* in the treatment of osteoporosis. Previous investigation by Yao *et al.*[29] showed that *C. chinensis* significantly promoted cell proliferation and differentiation, while four different traditional Chinese medicines, from *Eucommia ulmoides*, *Dipsacus asper*, *Loranthus parasiticus*, and *Achyranthes bidentata*, had no osteogenic effects. Although the osteogenic effects of *C. chinensis* have been elucidated, To-Sa-Za's effects on differentiation and mineralization of osteoblasts were unknown. To our knowledge, this is the first demonstration that TSZ-AE can regulate much of the tightly linked control between maturation and differentiation in MG-63 cells, through increased synthesis and secretion of growth factor and matrix proteins, and ultimately stimulates mineralization.

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## AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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