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Effect of Extracts from Safflower Seeds on Osteoblastic Differentiation and Intracellular Free Calcium Concentration in MC3T3-E1 Cells

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Very little research has been carried out on safflower seed for the prevention and treatment of the bone deficiency diseases, including osteoporosis, which are supported by scientific evidences. In the present study, 3 μl of 0.1% dried crude extract or 2 μl of 0.1% dried aqueous fraction were shown to significantly accelerate the rate of differentiation of osteoblast. Also, the crude extract and aqueous fraction increased the $[Ca^{2+}]_i$ of the cultured osteoblast cells: 3 μl of 0.1% dried crude extract and 2 μl of 0.1% dried aqueous fraction significantly increased the $[Ca^{2+}]_i$ of the cultured osteoblast cells (8×10^4) to the extent that it deserves a considerable attention. Furthermore, the crude extract and aqueous fraction increased the $[Ca^{2+}]_i$ of the cultured osteoblast cells, and 300 μM Cd^{2+}, specific calcium channel blocker, completely blocked the increase. Therefore, the increased $[Ca^{2+}]_i$ of the cultured osteoblast cells by safflower seed component continued to activate calcium channel.

Key Words: Safflower seed extracts, Intracellular free calcium concentration, MC3T3-E1 cells, Osteoblastic differentiation, Alkaline phosphatase activity

INTRODUCTION

Recently, natural herb medicines have been evaluated for their effects on periodontal diseases, such as, antibacterial and anti-inflammatory effects, and periodontal tissue regeneration (Akihisa et al, 1996; Kim et al, 2002b). Safflower (*Carthamus tinctorius L.*), an annual herbaceous plant belonging to a composite of chrysanthemum, has been traditionally used in oriental medicines to promote blood circulation, resolve blood stasis, and control pain (Kutsuna et al, 1988).

Safflower seeds in Korea have long been clinically used as herbal medicine to promote bone formation and to prevent osteoporosis (Huh, 1989; Huh et al, 2001): It was reported that safflower seeds have a protecting effect on bone loss caused by estrogen deficiency, without substantial effect on the uterus (Kim et al, 2002a). However, very little research have been carried out on the safflower seed for the prevention and treatment of the bone deficiency disease, including osteoporosis, which have been supported by scientific evidences.

The aim of this research was two fold: (i) to provide a basis for studying the mechanism of pharmacological action of safflower seed and (ii) to provide basic materials for designing new drug development which can prevent and cure bone deficiency diseases, including osteoporosis, with minimum side and toxic effects at a remarkably low cost with high efficacy. In the present study, we investigated the effects of the crude extract from Korean safflower seeds extracted by methyl alcohol and an aqueous fraction separated from the crude extract on osteoblastic differentiation and intracellular free calcium ion concentration $\{[Ca^{2+}]_i\}$ in MC3T3-E1 cells. We also determined the activity of alkaline phosphatase (ALP) and analyzed the expression of bone-related nodule formation by using histochemical methods.

METHODS

Materials

The mouse osteoblastic cell line, MC3T3-E1, was donated by Prof. Hyun-Mo Ryoo (School of Dentistry Kyungpook National University, Taegu, Korea). Safflower seeds were purchased from Safflower Seed Company at Uisung (Kyunpook, Korea). α-Minimum essential medium (α- MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA), 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzo-furan-5-oxy]-2-(2'-amino-5'-

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracelluar free calciumion concentration; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzo- furan-5-oxy]-2-(2'-amino-5'-methyl-phenoxy)-ethane-N,N,N',N'-tetra-acetic acid penta-acetoxymethyl ester.

methyl-phenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid penta-acetoxymethyl ester (Fura-2/AM) from Molecular Probes (Eugene, OR, USA), and thapsigargin and 4-bromo-calcium ionophore (A23187) from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of the highest purity available and were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated.

Cell culture

Cells were cultured at 37°C and 5% CO_2 atmosphere in Modified Eagle's Medium (α-MEM) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sudo et al, 1983; Kodama et al, 1994; Lasson et al, 2002). Cells were seeded at initial density of 2×10^4 cells/cm^2 and were grown in 10% FBS containing 10 mM β-glycero-phosphate (β-GP, Sigma, St. Louis, MO, USA) and 50 μg/ml of ascorbic acid with or without the crude extract (or aqueous fraction) in 100 mm dishes or 24-well plates (Corning, Corning, NY). The medium was changed every 3 days.

The cells cultured by the aforementioned technique were designated as the control group. The cells cultured in the culture solution containing some crude extract from safflower seeds were designated as the experimental group I, and in the medium with aqueous fraction from the seeds as experimental group II.

Histochemical analysis

To assess the time of nodule formation, cells were cultured in 100 mm dishes and maintained for 30 days. To detect calcium deposits, cells were rinsed twice with the ice-cold phosphate buffered saline (PBS) and incubated for 1 h in 0.1% Alizarin Red S solution at room temperature. Cells were stained with 0.1% light green SF solution for 30 min. Before examining under a light microscopy, cells were serially washed with 1% acetic acid and absolute ethanol (Dahl, 1952).

Determination of alkaline phosphatase activity (ALP)

Cells were cultured in 24-well plates at a density of approximately 4×10^4 cells per well. The medium was changed every 3 days during the culture period for up to 30 days. Cells were then washed with PBS and lysed in 1 ml of 0.02% Nonidet P-40 (Sigma, St Louis, MO, USA). The lysates were sonicated for 15s with an ultrasonicator (Sonic & Materials, VC375, USA) adjusted for 30% output. The sonicated lysates were centrifuged at 12,000g for 15 min. The supernatant was kept at −20°C before the assay was performed. The activity of ALP in lysates was measured by using ρ-nitrophenyl phosphate as the substrate, and optical density at 410 nm was determined as previously described (Bessay et al, 1946). Protein concentration was estimated by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The activity of ALP was expressed as nmol/min/mg of protein.

Fluorescence optical measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$-measurements were carried out as described previously (Lewis & Cahalan, 1989; Verheugen et al. (1997). Briefly, Fura-2/AM loading was as described by (Thomas & Delaville, 1991), and solubilized cells were incubated at 37°C for 20 min in 3 ml.

To remove the culture medium, cells were washed three times with Hepes-buffered saline (HBS-control) containing (concentration in mol/l) NaCl 140, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11 and Hepes 10, pH 7.4. Stock solutions of crude extract or aqueous fraction from safflower seeds were added to this saline shortly before the experiments. Measurements of $[Ca^{2+}]_i$ were carried out with a Ratio Fluorescence Spectrometer (PTI, RI-D, USA) with ratios being collected at 0.05~1 Hz, equipped with a thermostated cell holder, and performed at pH 7.4 (37± 0.1°C).

Cells having a total volume of 3 ml were placed in a quarts cuvette and slowly stirred. Recordings were made for 2 periods of 300~600s. Ethyleneglycol-bis(-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) was not used to buffer external Ca^{2+}, since it strongly binds heavy metals.

Relative changes of $[Ca^{2+}]_i$ were calculated from the excitation ratios (R; excitation 340/380 nm; emission 510 nm, collected for background fluorescence). Absolute $[Ca^{2+}]_i$ in MC3T3-E1 cells (8×10^4) was estimated according to the following equation of Grynkiewicz et al. (1985).

$$[Ca^{2+}]_i = K_d \cdot S_f\,(R-R_{min})/(R_{max})-R$$

Where K_d is the dissociation constant of Fura-2 (assumed to be 350 nM) (Negulescu & Machen, 1990), S_f is a device dependent scale factor and R is the ratio of the two measured fluorescence intensities, 340/380 nm. R_{min} and R_{max} were determined in vitro using solutions containing zero Ca^{2+} and 39.8 μM Ca^{2+}, respectively (Fura-2 calcium imaging calibration kit, Molecular Probes, Eugene, OR, USA).

Data analysis

Statistical comparisons were performed using one-way analysis of variance followed by Student's Newman-Keuls post hoc test, when comparing a factor with more than two levels. The test used was two-tailed, and the significance level was set at $P<0.05$ and $P<0.01$. Data are presented

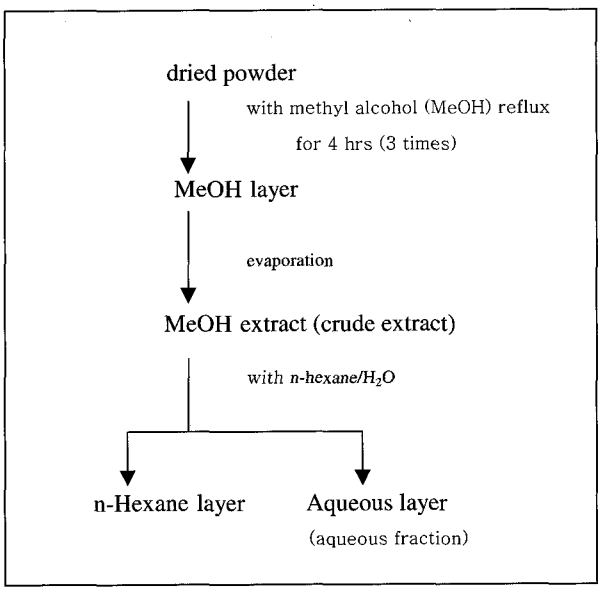

Fig. 1. Schematic diagram of extraction of crude extract and aqueous fraction from safflower seeds.

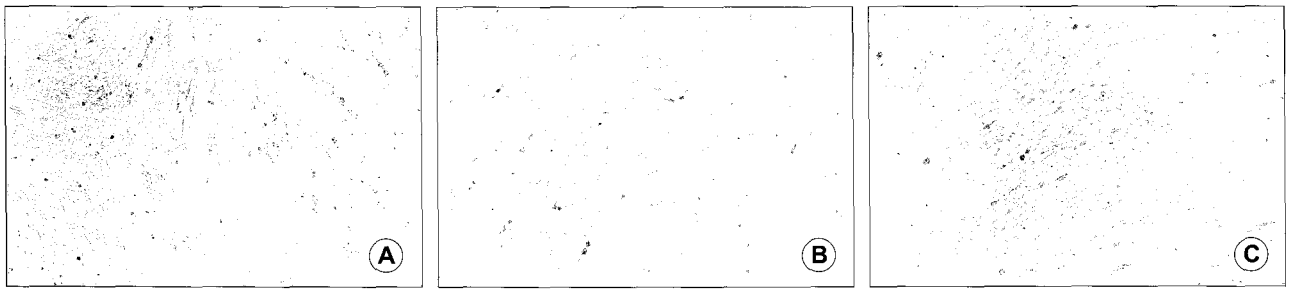

Fig. 2. Histochemical changes during differentiation of MC3T3-E1 osteoblastic cells. Phase contrast micrographs (×40) were taken by Alizarin Red S staining after 5 days. (A) Control (absence of the seed component in the culture solution), (B) Experimental group I (added 3 μl of 0.1% dried crude extract in the culture solution), (C) Experimental group II (added 2 μl of 0.1% dried aqueous fraction in the culture solution).

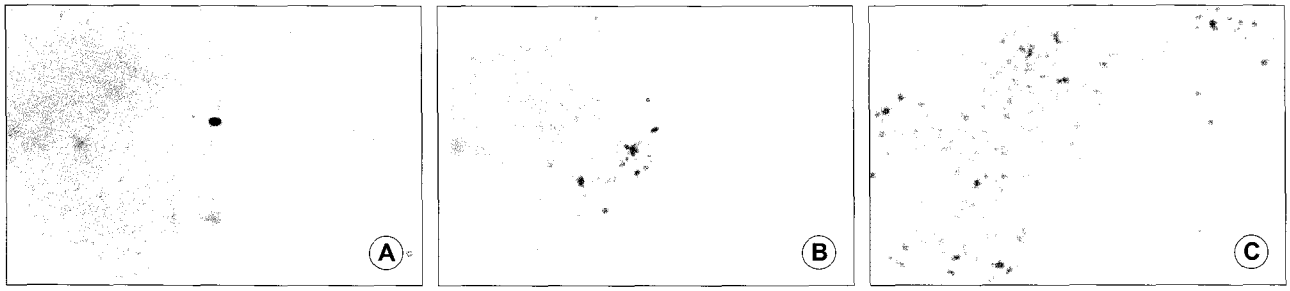

Fig. 3. Histochemical changes during differentiation of MC3T3-E1 osteoblastic cells. Phase contrast micrographs (×40) were taken by Alizarin Red S staining after 10 days. (A) Control (absence of the seed component in the culture solution), (B) Experimental group I (added 3 μl of 0.1% dried crude extract in the culture solution), (C) Experimental group II (added 2 μl of 0.1% dried aqueous fraction in the culture solution).

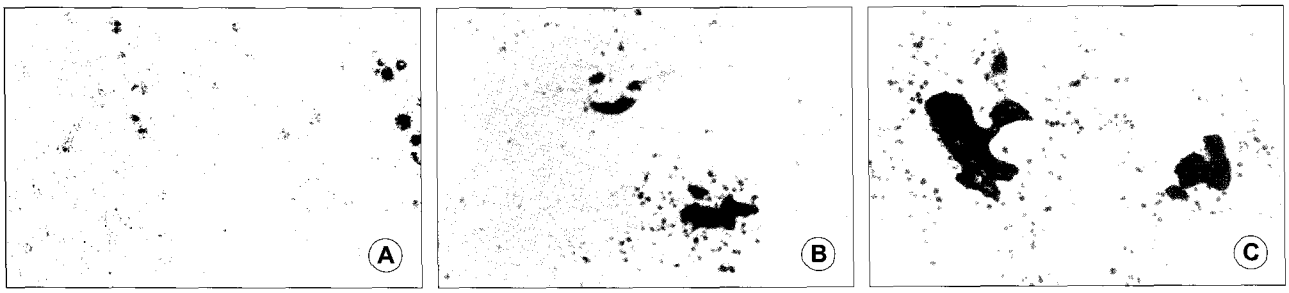

Fig. 4. Histochemical changes during differentiation of MC3T3-E1 osteoblastic cells. Phase contrast micrographs (×40) were taken by Alizarin Red S staining after 15 days. (A) Control (absence of the seed component in the culture solution), (B) Experimental group I (added 3 μl of 0.1% dried crude extract in the culture solution), (C) Experimental group II (added 2 μl of 0.1% dried aqueous fraction in the culture solution).

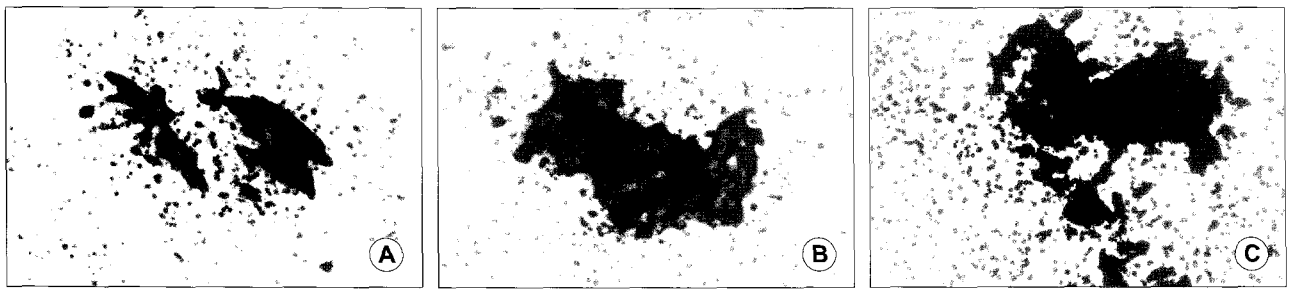

Fig. 5. Histochemical changes during differentiation of MC3T3-E1 osteoblastic cells. Phase contrast micrographs (×40) were taken by Alizarin Red S staining after 30 days. (A) Control (absence of the seed component in the culture solution), (B) Experimental group I (added 3 μl of 0.1% dried crude extract in the culture solution), (C) Experimental group II (added 2 μl of 0.1% dried aqueous fraction in the culture solution).

as mean±SEM (n=7 for all experiments).

Isolation of crude extract and aqueous fraction from safflower seeds

Whole Safflower seeds were cracked, dehulled, flaked and powdered by standard procedure as described Fig. 1.

RESULTS

Nodule formation during the MC3T3-E1 cell differentiation

Bone nodule formation is one of the markers of the bone cell differentiation. To determine the time of nodule formation, cells were stained with Alizarin Red S at the indicated time (Fig. 2~5). As shown in Fig. 2, the nodules were not observed after 5 days in this culture system, however, the indications of nodules were observed and the magnitude and number of the indications of nodules decreased in the order of the experimental group II (Fig. 2C), experimental group I (Fig. 2B) and control group (Fig. 2A). The nodules were observed after 10 days in this culture system (Fig. 3) and the number of Alizarin Red S positive nodules increased during the MC3T3-E1 cell differentiation (Fig. 3~5). In general, depending on the presence or absence of the seed extract in the culture system, the magnitude and number of Alizarin Red S positive nodules differed in the descending order of the experimental group II (Fig. 3C~5C), experimental group I (Fig. 3B~5B) and control group (Fig. 3A~5A). To confirm that this culture condition was important for the formation of the bone nodules, MC3T3-E1 cells were cultured for 30 days in α-MEM containing 10% FBS without ascorbic acid and β-GP, and no Alizarin Red S positive nodules were observed (data not shown). We also used the same serum lot of FBS to rule out the effects of serum variation. The increase of the nodule formation as the function of time course indicates that this culture condition is favorable to show the expression patterns of bone-related proteins.

Alkaline phosphatase activity during the MC3T3-E1 cell differentiation

As another marker of the osteoblast differentiation, we determined the activity of ALP during the MC3T3-E1 cell differentiation. The activity of ALP increased significantly and reached the maximal level at around day 9 and subsequently dropped to a level that was still higher than that of day 4 (Fig. 6), indicating that the maximal level of the ALP activity was before the expression of the nodules formation. This pattern of ALP activity is similar to the rat calvarial osteoblasts (Bronckers et al, 1987; Aronow et al, 1990; Owen et al,. 1990; Choi et al, 1996). Histochemical analysis revealed that the magnitude of the ALP activity differed, depending on the presence or absence of the seed extract in the cultural solution, in the descending order of the experimental group II, experimental group I and control group (Fig. 6).

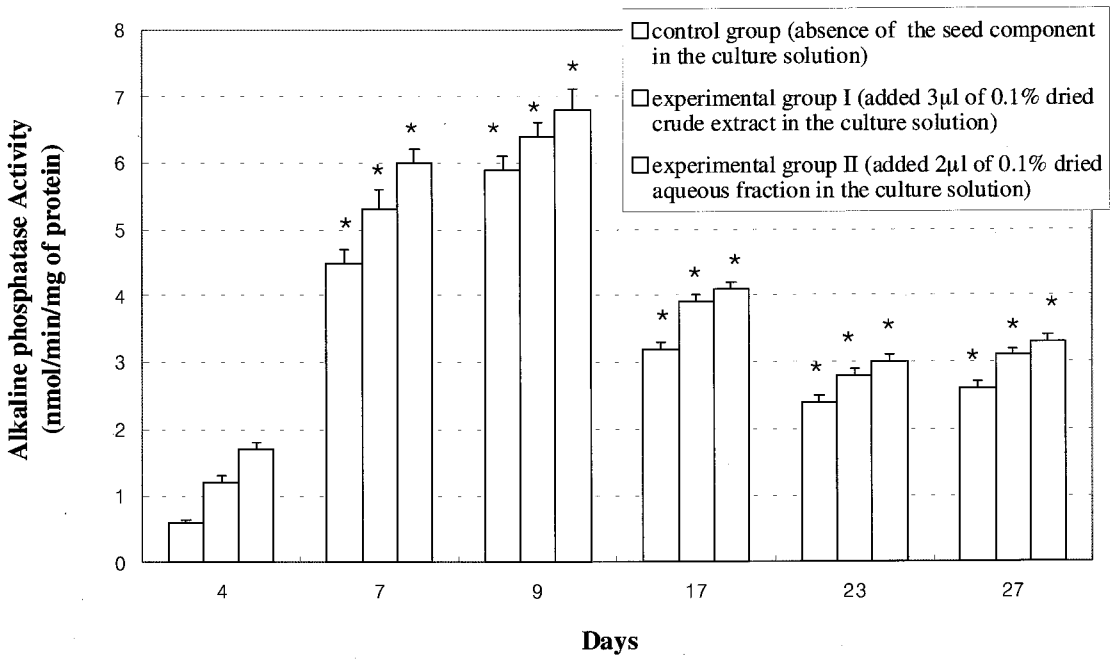

Fig. 6. Effects of crude extract or aqueous fraction from safflower seeds on alkaline phosphatase (ALP) activity during the differentiation of the MC3T3-E1 osteoblastic cells. Cells were cultured in 24 well plates with 50 μg/ml of ascorbic acid and 10 mM β- glycerophosphate. ALP activity in cell layers was determined at the indicated times (4, 7, 9, 17, 23, 27 days) described in Materials and Methods. The medium was changed every 3 days. Data were expressed as mean±SEM of seven determinations. An asterisk signifies $P<0.05$ according to Student's Newman-Keuls post hoc test.

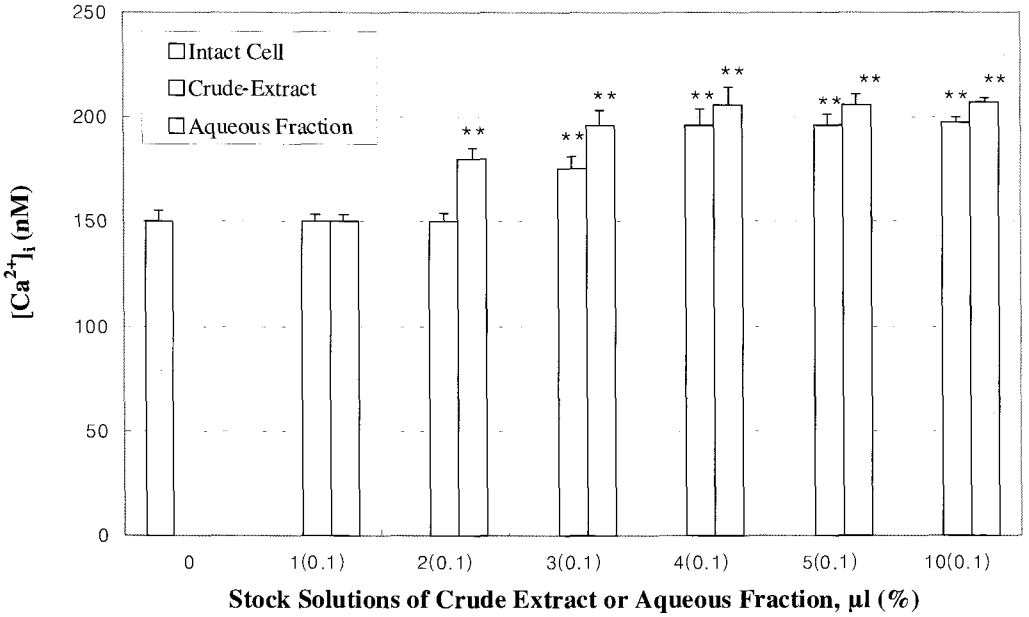

Fig. 7. Effects of crude extract or aqueous fraction from safflower seeds on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the MC3T3-E1 cells (8×10^4). The cells were washed three times with Hepes-buffered saline (HBS-control) containing (concentration in mol/l) NaCl 140, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11 and Hepes 10, pH 7.4. Stock solutions of 0.1% crude extract or aqueous fraction were added to this saline shortly before determinations. Determinations were performed at 37±0.1°C (pH 7.4). Each value represents mean±SEM of 7 determinations. Double asterisk signifies $P<0.01$ according to Student's Newman- Keuls post hoc test.

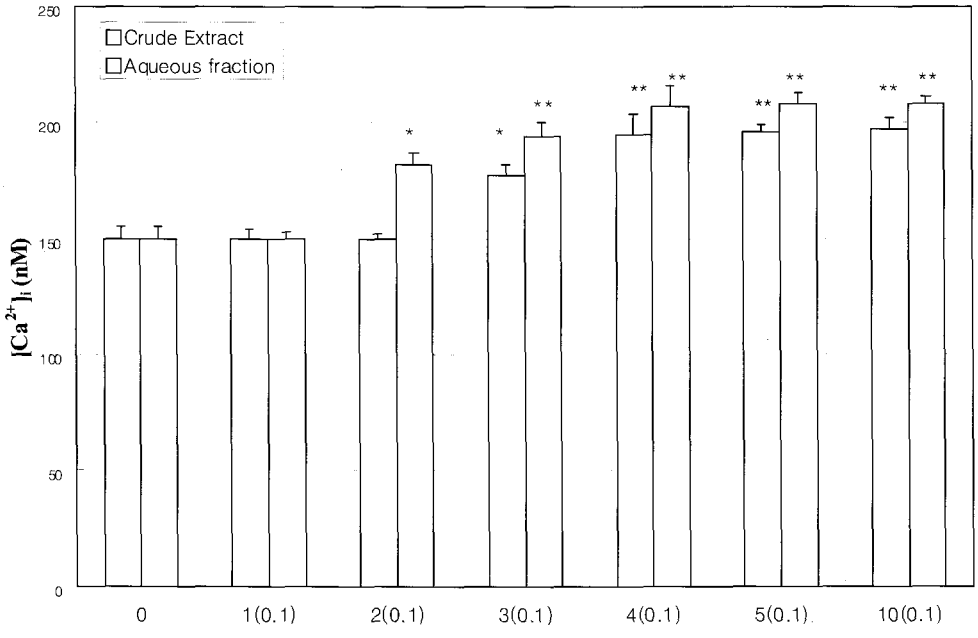

Fig. 8. Effect of 400 μM A23187 on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the MC3T3-E1 cells (8×10^4). The cells were washed three times with Hepes-buffered saline (HBS-control) containing (concentration in mol/l) NaCl 140, KCl 3, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11 and Hepes 10, pH 7.4. The cells were preincubated for 10 min in 400 μM A23187 and Ca^{2+} free HBS. And then added 3 μl of 0.1% dried crude extract (or 2 μl of 0.1% aqueous fraction) in the HBS. Stock solutions of 0.1% crude extract or aqueous fraction were added to this saline shortly before determinations. Determinations were performed at 37±0.1°C (pH 7.4). Each value represents mean±SEM of 7 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to the control according to Student's Newman- Keuls post hoc test.

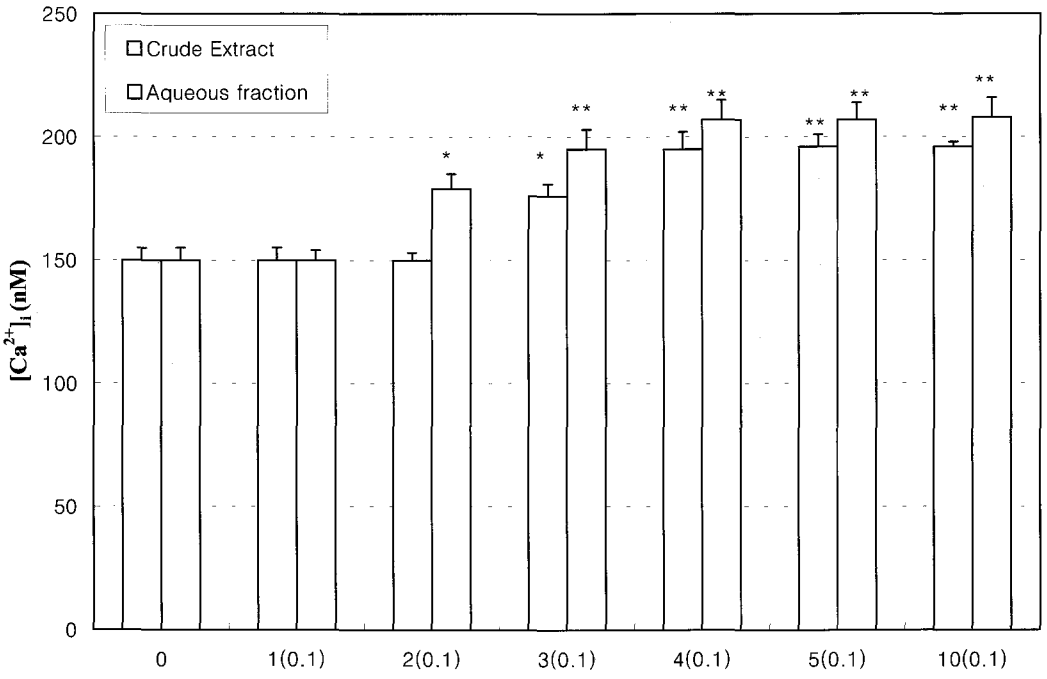

Fig. 9. Effect of 1 mM thapsigargin on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the MC3T3-E1 cells (8×10^4). The cells were washed three times with Hepes-buffered saline (HBS-control) containing (concentration in mol/l) NaCl 140, KCl 3, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11 and Hepes 10, pH 7.4. The cells were preincubated for 10 min in 1 mM thapsigargin and Ca^{2+} free HBS. And 3 μl of 0.1% dried crude extract (or 2 μl of 0.1% aqueous fraction) were then added in the HBS. Stock solutions of 0.1% crude extract or aqueous fraction were added to this saline shortly before determinations. Determinations were performed at $37\pm0.1^{\circ}C$ (pH 7.4). Each value represents mean$\pm$ SEM of 7 determinations. An asterisk and double asterisk signify $P<0.05$ and $P<0.01$, respectively, compared to the control according to Student's Newman- Keuls post hoc test.

Effects of crude extract and aqueous fraction from safflower seeds on intracellular free calcium concentration of MC3T3-E1

In the present study using the fluorescence probe technique, the effect of both crude extract and aqueous fraction from safflower seeds on $[Ca^{2+}]_i$ of MC3T3-E1 cells (8×10^4 cells) was examined. $[Ca^{2+}]_i$ of intact cells was 150 ± 5 nM. As shown in Fig. 7, both crude extract and aqueous fraction increased the $[Ca^{2+}]_i$ of MC3T3-E1 cells in a concentration-dependent manner: The significant increase in the $[Ca^{2+}]_i$ of MC3T3-E1 cells by aqueous fraction and crude extract was observed at such low concentrations as 2 μl of 0.1% and 3 μl of 0.1% in the extracelluar solution, respectively (Fig. 7). It was significantly increased compared to the $[Ca^{2+}]_i$ in the absence of safflower seed extracts in the extracellular solution.

DISCUSSION

The biochemical and histochemical data obtained in this study indicate that the expression patterns, including proliferation, bone matrix formation/maturation, and mineralization which are regulated in a temporal manner during successive developmental stages, are unique in the MC3T3-E1 cell culture system, compared with other systems.

The existence of this stage during the MC3T3-E1 cell differentiation is manifested by the formation of the nodules. This period is called the mineralization stage. The nodule formation is observed only for the late mineralization stage (days 10~30) of this culture system. This result confirms independent observation of previous studies in MC3T3-E1 (Sudo et al, 1983; Franceschi & Lyer, 1992; Dean et al, 1994) and several other cells (Gerstenfeld et al, 1987; Bellows et al, 1991; Stein & Lian, 1993; Malaval et al, 1994). In addition, the nodule formation could not be observed when cells were cultured without ascorbic acid and β-GP, in good agreement with other reports (Franceschi & Lyer, 1992; Dean et al, 1994). This finding is in accordance with the results of studies in the rat calvarial osteoblasts (Bronckers et al, 1987) and the bone marrow cells (Malaval et al, 1994). These differences may partly result from the difference of the culture conditions such as cell plating number and serum lot.

The MC3T3-E1 cell culture system represents a very useful model for studying the developmental regulation of the bone-related proteins in relation to the different stages during the osteoblast differentiation and the molecular mechanisms associated with the bone formation. The present research has proved that the elements of safflower seed stimulate the influx of Ca^{2+} from the extracellular fluid to the intracellular fluid (Fig. 7) by activating the calcium channel of the osteoblast cells (Fig. 8~10), thereby

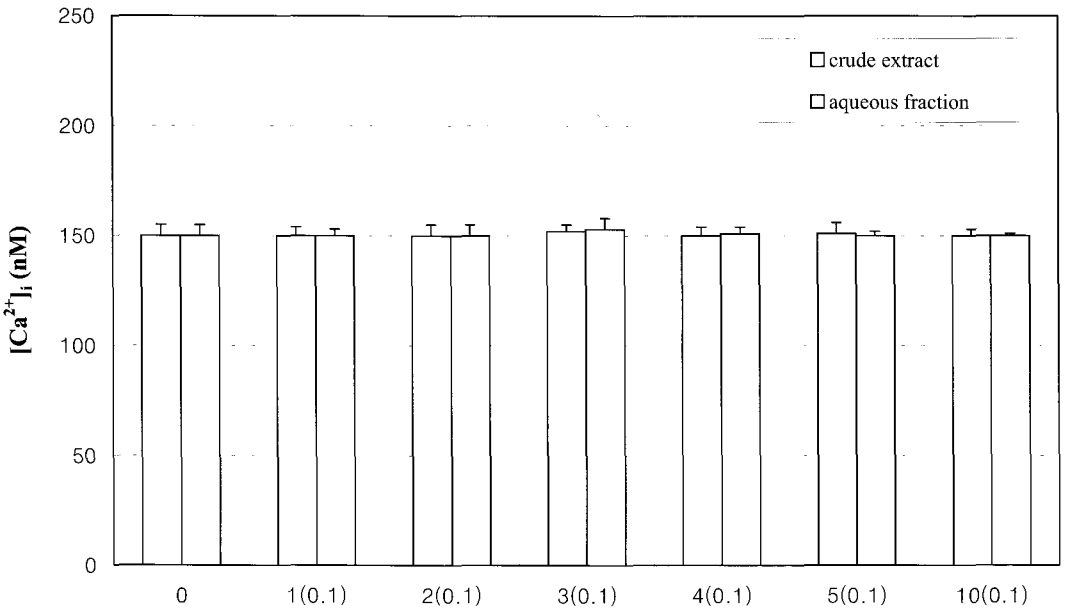

Fig. 10. Effect of 300 μM $CdCl_2$ on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the MC3T3-E1 cells (8×10^4). The cells were washed three times with Hepes-buffered saline (HBS-control) containing (concentration in mol/l) NaCl 140, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 1.3, KH_2PO_4 1.25, glucose 11 and Hepes 10, pH 7.4. The $[Ca^{2+}]_i$ was evaluated in the presence of 300 μM $CdCl_2$ plus crude extract (or aqueous fraction) in extracellular solution (HBS). Stock solutions of 0.1% crude extract or aqueous fraction were added to this saline shortly before determinations. Determinations were performed at 37±0.1°C (pH 7.4). Each value represents mean±SEM of 7 determinations.

increasing the free Ca^{2+} of intracellular fluid and accelerating the rate of osteoblast cell differentiation in culture.

In the present study, samples were crude extract and aqueous fraction isolated from Korean safflower seeds. MC3T3-E1 cells cultured in the presence of 3 μl of 0.1% dried crude extract or 2 μl of 0.1% dried aqueous fraction had significantly faster and more clear growth of nodules and had a bigger number of nodules compared to the cells cultured in the absence of these components. Therefore, we conclude that the crude extract and aqueous fraction increased the rate of differentiation of MC3T3-E1 cells.

MC3T3-E1 cells cultured in the presence of 3 μl of 0.1% dried crude extract or 2 μl of 0.1% dried aqueous fraction had significantly higher ALP activity (Fig. 6), compared to the cells cultured in the absence of the extracts, suggesting that the extracts increased the rate of differentiation and proliferation of the osteoblastic cells

This study also demonstrated that the crude extract and aqueous fraction from safflower seeds elevated $[Ca^{2+}]_i$ in the MC3T3-E1 cells. The increase in $[Ca^{2+}]_i$ observed in the osteoblast-like cell line (MC3T3-E1 cells) was rapid, transient and dose-dependent, thus indicating that the increase in $[Ca^{2+}]_i$ in osteoblastic cells represents an influx from extracellular solution to intracellular fluid, with aqueous fraction being more effective (Fig. 7).

The cytoplasmic (intracellular) $[Ca^{2+}]_i$ can be elevated by either opening of plasma membrane Ca^{2+}-channels or through release from intracellular stores. The plasma membrane has a variety of Ca^{2+} entry channels that can be distinguished by their mode of activation. Voltage-gated Ca^{2+}-channels open in response to membrane depolarization to mediate a selective Ca^{2+} entry, whereas receptor-operated calcium channels open in response to interaction with specific agonists. The major intracellular Ca^{2+} stores that can release its content in response to hormonal stimulation is the endoplasmic reticulum (Tsien & Tsien, 1990). This release is controlled by two families of channels: ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptors, IP_3R (Berridge, 1993).

To find out whether the increase $[Ca^{2+}]_i$ was due to Ca^{2+} entering the cells from extracellular solution or Ca^{2+} release from internal stores, the cells in Ca^{2+} free HBS were preincubated for 10 min with 400 μM A23187 or 1 mM thapsigargin in HBS: Thapsigargin and A23187 have depleting effect of calcium stores in the endoplasmic reticulum. We found that the increase of $[Ca^{2+}]_i$ was still maintained, leading us to the conclusion that the increase of $[Ca^{2+}]_i$ was not due to the release of Ca^{2+} from the intracellular store site, but due to the influx of Ca^{2+} from the extracellular solution to the intracellular fluid by components of safflower seed extracts (Fig. 8, 9).

When $CdCl_2$ (300 μM), a specific Ca^{2+}-channel blocking agent was added to the extracellular solution to further confirm whether the increase of $[Ca^{2+}]_i$ was due to an influx of Ca^{2+} through Ca^{2+}-channel by safflower seed extracts, the increase of $[Ca^{2+}]_i$ was blocked. Thus, we were able to further confirm that the increase of $[Ca^{2+}]_i$ was due to an influx of Ca^{2+} through the Ca^{2+}-channel from extracellular solution to the intracellular fluid by the action of safflower seed extracts (Fig. 10).

Based on the present results, the reason of why Koreans obtained favorable results by administering safflower seeds

in treating bone deficiency diseases became very clear: safflower seeds have an effect of facilitating the influx of Ca^{2+} from the extracellular fluid to the intracellular fluid. Indeed, the effects of safflower extracts on bone formation, preventino of osteoporosis (Huh et al, 2001) and protection of bone loss (Kim et al, 2002a) are partially attributed to the fact that safflower extracts activate Ca^{2+}-channel of osteoblastic cells.

We strongly believe that continuous research on the components of safflower seed should be conducted in order to further confirm their efficacy and chemical structure, thus providing theoretical basis for launching into a mass production system.

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