Chinese Herbal Medicines 13 (2021) 534–540

Contents lists available at ScienceDirect

Chinese Herbal Medicines

journal homepage: www.elsevier.com/locate/chmed

Original Article

High water-soluble curcuminoids-rich extract regulates osteogenic differentiation of MC3T3-E1 cells: Involvement of Wnt/β-catenin and BMP signaling pathway

Yutthana Pengjam [a], Nurul Syazwani [b], Jakkapong Inchai [a], Amornkan Numit [a], Thanintorn Yodthong [c], Thanawat Pitakpornpreecha [c], Pharkphoom Panichayupakaranant [b,*]

[a] Faculty of Medical Technology, Prince of Songkla University, Hat-Yai 90110, Thailand
[b] Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai 90112, Thailand
[c] Faculty of Science, Prince of Songkla University, Hat-Yai 90110, Thailand

ARTICLE INFO

Article history:
Available online 26 January 2021

Keywords:
BMP signaling
curcuminoid
osteoporosis
solid dispersion
water soluble
Wnt/β-catenin

ABSTRACT

Objective: The present study aimed to evaluate the effect of a high water-soluble curcuminoids-rich extract (CRE) in a solid dispersion form (CRE-SD) using polyvinylpyrrolidone K30 on osteogenic induction of MC3T3-E1 cells.

Methods: CRE was pre-purified using a microwave assisted extraction couple with a Diaion® HP-20 column chromatography. The osteoblastic cell proliferation and differentiation potentials of CRE-SD in MC3T3-E1 cells were tested by cell viability, alkaline phosphatase (ALP) activity, and Alizarin red S activity assays. The mRNA expressions of osteoblast-specific genes and underline mechanisms were assessed by a real time PCR and western blot analysis.

Results: CRE-SD 50 μg/mL increased alkaline phosphatase (ALP) activity, an early differentiation marker of osteoblasts in both MC3T3-E1 cells and non-osteogenic mouse pluripotent cell line, C3H10T1/2, indicating the action of CRE-SD was not cell-type specific. Alizarin red S activity showed a significant amount of calcium deposition in cells treated with CRE-SD. CRE-SD also upregulated the mRNA expression levels of transcription factors that favor osteoblast differentiation including Bmp-2, Runx2 and Collagen 1a, in a dose dependent manner. Western blot analysis revealed that noggin attenuated CRE-SD-promoted expressions of Bmp-2 and Runx2 proteins. siRNA mediated blocking of Wnt/β-catenin signaling pathway also annulled the influence of CRE-SD, indicating Wnt/β-catenin dependent activity. Inhibition of the different signaling pathways abolished the influence of CRE-SD on ALP activity, confirming that CRE-SD induced MC3T3-E1 cells into osteoblasts through Wnt/β-catenin and BMP signaling pathway.

Conclusion: These results collectively demonstrate that CRE-SD may be a potential therapeutic agent for the treatment of osteoporosis.

1. Introduction

Osteoporosis is a bone disease common in the aging population and among post-menopausal women. It is characterized by low bone mass and micro-architectural deterioration of bone tissue, rendering the individual highly susceptible to fragility fractures (Deschaseaux, Sensebe, & Heymann, 2009). Anti-resorptive agents that inhibit osteoclastic bone resorption and anabolic agents that stimulate osteoblastic bone formation, have been used to treat such a disease. Anti-resorptive agents such as bisphosphonates, selective estrogen receptor modulators and calcitonin are currently available for the treatment of osteoporosis (Romas, 2005). However, these anti-resorptive agents have disadvantages, such as causing unusual fractures in the femur and shaft of the bone and causing flu-like symptoms (Feldbrin Luckish, & Shargorodsky, 2016).

Plant-derived natural compounds, such as icariin (Chen et al., 2005), genistein (Sugimoto & Yamaguchi, 2000), epigallocatechin-3-gallate (Vali, Rao, & Elsohemy, 2007), resveratrol (Mizutani, Ikeda, Kawai, & Yamori, 1998), harmine (Yonezawa et al., 2011), and aloin (Yonezawa et al., 2016) have been found to stimulate osteoblast differentiation and bone formation. These compounds could serve as useful anabolic agents. However, limited availability of natural agents to enhance bone mass is still a source of concern.

* Corresponding author.
E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant).

https://doi.org/10.1016/j.chmed.2021.01.003
1674-6384/

Turmeric (*Curcuma longa* L.) is grown throughout Thailand and other Southeast Asia countries. The major active compounds of turmeric are curcuminoids, namely curcumin (Cu), demethoxycurcumin (De), and bis-demethoxycurcumin (Bis) (Woo et al., 2003). Curcumin has been used to cure osteoporosis (Folwarczna, Zych, & Trzeciak, 2010) due to the inhibition of osteoclastogenesis (Bharti, Takada, & Aggarwal, 2004; Kim et al., 2011; Martins, Leyhausen, Volk, & Geurtsen, 2015). Although curcuminoids are extremely safe, tolerable, and nontoxic in animal and human studies even at very high doses (Shoba et al., 1998; Cheng et al., 2001; Lao et al., 2006), they are not approved as therapeutic agents because of their limited solubility in the human gastrointestinal tract, limited gastrointestinal absorption (Anand et al., 2008) and rapid metabolism both in the intestines and the liver (Ireson et al. 2002). An increase in solubility of cucuminoids resulted in an increased dissolution as well as oral bioavailability. Solid dispersion is a method that successfully used to improve the solubility, dissolution and consequently the oral bioavailability of poorly soluble drugs (Okonogi et al., 1997). It has been reported that solubility and dissolubility of curcumin were successfully increased by solid dispersion using polyvinylpyrrolidone K30 (PVP K-30) as a hydrophilic carrier (Kaewnopparat et al., 2009).

MC3T3-E1 cells possess a very high osteoblast differentiation potential, expressing osteoblast phenotypic marker genes and mineralizing after the addition of suitable inducible factors (Muhammad et al., 2010; Wang et al., 2011).

Bone morphogenetic proteins (Bmp) are potent inducers of osteoblastogenesis and the crucial protein in BMP signaling pathway. Bmp activate the transcription factor, Runt-related transcription factor 2 (Runx2), which translocate into the nucleus and modulates the expression of many target genes (Chen, Zhao, & Mundy, 2004). Collagen 1a and Osteopontin are also known as essential proteins for osteoblast differentiation. Another important junction of osteoblast differentiation is the Wnt family, which plays an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis (Baron & Kneissel, 2013).

The present study aimed to investigate the stimulating effects of the high water-soluble CRE in solid dispersion form (CRE-SD) on osteoblastic differentiation and its detailed mechanism of action using MC3T3-E1 cells, and determined the effect of CRE-SD on expression of osteoblast marker genes Bmp-2, Runx2, Collagen 1a, and Osteopontin, and the effect of CRE-SD on BMP and Wnt/β-catenin signaling pathways. Furthermore, the effects of blockade of BMP signaling pathway by antagonist noggin and Wnt/β-catenin signaling pathway using small interfering RNA (siRNA) were also investigated.

2. Materials and methods

2.1. Preparation of CRE-SD

CRE-SD (containing 7% curcuminoids) was prepared using the methods previously described (Lateh, Yuenyongsawad, Chen, & Panichayupakaranant, 2019; Lateh et al., 2018). Briefly, the dried turmeric powders were extracted with ethanol using the microwave-assisted extraction conditions as follows: power of 900 Watt, at 70–75 °C, with three irradiation cycles (one cycle = 3 min power-on, and 30 s power-off). The extract was filtered and then subjected to a Diaion® HP-20 column eluted with 55% and 60% (volume percent) ethanol, respectively to obtain curcuminoids enriched extract (CRE). CRE-SD was prepared using a solvent evaporation method. CRE was dispersed in PVP K30 (10%, mass ratio), and subsequently solvent evaporated under reduced pressure to obtained CRE-SD powders. Based on HPLC analysis, CRE-SD contained 7% w/w curcuminoids.

All the chemicals were obtained from Sigma (St. Louis, MO, USA) or Wako Pure Chemical Industries Ltd.

2.2. Cell cultures

MC3T3-E1 cells were obtained from the Cell Bank (Germany). Cells were cultured in α-MEM cell culture medium (Gibco Co., Bangkok, Thailand) with 10% FBS and anti-bacterial cocktail (PNS), and incubated in a humidified chamber (5% CO_2, 37 °C). At semi confluence stage, the cells were treated with different concentrations of CRE-SD for 10 d or 3 weeks, as required.

2.3. Cell viability and cell cytotoxicity

MC3T3-E1 cells seeded at a density of (0.3×10^3) cells/mL in a 96-well plate were treated with CRE-SD at different concentrations for 10 d and subjected to MTT and LDH assays for cell viability and cell cytotoxicity, respectively. For the MTT experiment, the yellow tetrazolium MTT was reduced by metabolically active cells into an insoluble formazan salt. The resulting intracellular purple formazan was solubilized and further quantified with a spectrophotometer (Thermo scientific, Multiskan FC, Pittsburgh PA, USA) at a wavelength of 570 nm. A non-radioactive cytotoxicity assay kit (CytoTox 96®) was used for LDH assays. After incubating cells with lactate dehydrogenase enzyme buffer for 1 h, the colorimetric measurement of cytosolic enzyme released into the medium upon cell lysis was measured using the spectrophotometer at a wavelength of 490 nm.

2.4. Cell proliferation assay

MC3T3-E1 cells were seeded at an initial density of (0.3×10^3) cells/mL in a 96-well plate and treated with 50 μg/mL of CRE-SD. After every other interval, cell proliferation assay was performed by the MTT method. An increase in cell number over the days was recorded.

2.5. ALP activity assay

MC3T3-E1 cells treated with CRE-SD for 10 d were washed twice with cold PBS and lysed in cell lysis buffer. ALP activity was assayed using an alkaline phosphatase staining kit (Primary Cell Co. Ltd, Tokyo, Japan). Briefly, lysates were incubated in ALP substrate buffer (100 mmol/L Tris-HCl pH 8.5, 2 mmol/L $MgCl_2$, 6.6 mmol/L 4-nitrophenyl phosphate) for 30 min. Absorbance at 405 nm was measured as the ALP activity using a microplate reader (Thermo scientific, Multiskan FC, Pittsburgh PA, USA).

2.6. Mineralization assay

Calcium deposition was measured using Alizarin red S staining. Briefly, cells were treated with CRE-SD for 21 d, and then washed with PBS and fixed with 10% formaldehyde for 15 min. Cells were stained with 40 mmol/L of Alizarin red S solution (pH 4.1–4.3) in the shaker (Labnet International, Big flats, NY, USA) at room temperature for 30 min. The non-specific staining was removed by washing with distilled water five times (5 min/time). Alizarin red S staining was dissolved by cetylpyridinium chloride for quantification and the absorbance was measured at 550 nm.

2.7. RNA isolation and real time PCR

Total RNA was isolated using ISOGEN (Nippon Gene, Toyama, Japan). cDNA was synthesized using ReverTra Ace qPCR kit (Toy-

obo, Osaka, Japan). RNA (1 μg) was used to synthesize first strand complementary DNA (cDNA) by using a miScript II RT kit (Qiagen, Inc., MD, USA). The first-strand cDNA was further used in conjunction with miScript primer assays and a SYBR green polymerase chain reaction (PCR) kit (Qiagen, Inc., MD, USA) for analysis of Bmp-2, Runx2, Collagen 1a and Osteopontin by real-time PCR, using ABI Prism 7500 HT sequence detection system (Applied Biosystems, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The relative expression levels of the target genes against GAPDH were calculated using an Image Quant TL (GE Healthcare Life Sciences, Japan) with a luminescent image analyzer (LAS 4000, Fujifilm, Japan).

Primers used were as follows.

Bmp-2; Forward: AGTTCTGTCCCCAGTGACGAGTTT
Reverse: GTACAACATGGAGATTGCGCTGAGRT

Osteopontin; Forward: TCACCATTCGGATGAGTCTG
Reverse: ACTTGTGGCTCTGATGTTCC

Runx2; Forward: CCGCACGACAACCGCACCAT
Reverse: CGCTCCGGCCCACAAATCTC

Collagen 1a; Forward: TTCCCTGGTGCTGATGGTGTTGCT
Reverse: GCCTTTCCAGGTTCTCCAGCGG

GAPDH; Forward: AAATGGTGAAGGTCGGTGTG
Reverse: GAATTTGCCGTGAGTGGAGT

2.8. Western blotting

Total cell lysates were prepared using RIPA buffer. Cytoplasmic and nuclear proteins were obtained using NE-PER reagent (Thermo-Scientific, Inc. Washington DC, USA) following manufacturer's instructions. Protein fractions were re-suspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) buffer containing 2-mercaptoethanol, and boiled at 95 °C for 5 min. Protein samples were subjected to SDS-PAGE in 10% polyacrylamide gel and subsequently electroblotted onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, NJ, USA). After blocking non-specific binding sites for 1 h in 3% nonfat milk in TBST (TBS and 0.1% Tween 20), membranes were incubated overnight at 4 °C with specific primary antibodies. Antibodies for β-catenin Wnt and β actin were purchased from Cell signaling (Massachusetts, USA). The membranes were washed with TBST and incubated further with horse-radish peroxidase-conjugated secondary antibodies at room temperature. Protein bands were detected using an enhanced ECL kit (GE Healthcare, Tokyo, Japan) with the digital imaging system (LAS4000).

2.9. Treatment with Bmp antagonist noggin

Subconfluent cells were treated with noggin (1 μg/mL) for 4 h, followed by 16 h incubation with CRE-SD 50 μg/mL. Cell lysates were prepared using RIPA buffer. Cell lysates were used for Western blot detection of Bmp-2 and Runx2. β-Actin was used as a housekeeping control.

2.10. Wnt siRNA transfection

Cells were plated into a 6-well plate with α-MEM cell culture medium with 10% FBS and anti-bacterial cocktail (PNS) at the humidified chamber (5% CO_2, 37 °C) as described previously. Wnt siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, Biotechnology, Inc, Texas, USA). siRNA transfection was performed according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were treated with 50 μg/mL CRE-SD. Cell lysates were analyzed for expression of Bmp-2 protein using the Western blotting, as described earlier (Pengjam et al., 2016).

2.11. ALP activity assay during pathway inhibitions

Subconfluent cells were treated with BMP antagonist (noggin), p38 MAPK inhibitor (SB), SAPK/JNK inhibitor (SP) and blockade of Wnt-inhibitor siRNA. For Wnt siRNA, 24 h post-transfection cells were treated with 50 μg/mL CRE-SD and then incubated for 10 d. ALP activity was assayed using the alkaline phosphatase staining kit, as described previously. Cells without CER-SD were used as control.

2.12. Culture of non-osteogenic mouse pluripotent cell line, C3H10T1/2

Non-osteogenic mouse pluripotent cell line, C3H10T1/2, were purchased from the Cell Bank (Germany). The cells were maintained in Eagle's basal medium (GIBCO, Thailand) containing 10% heat-inactivated fetal bovine serum (FBS). The initiation medium contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4, fetal bovine serum (10%), penicillin, streptomycin and amphotericin B. Cells were incubated under standard conditions (5% CO_2 and 37 °C) until they reached semi confluence. C3H10T1/2 from passage 2 were used for studies on osteogenesis differentiation by CRE-SD. Cells (8×10^4 cells/well) were treated with various concentrations of CRE-SD and ALP activity was determined as described earlier.

2.13. Statistical analysis

All data are expressed as mean ± SD. Statistical analyses of the significance of differences among values were carried out by one-way ANOVA with a post hoc Dunnett's test or Students T-test with $n = 4$ independent experiments. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results and discussion

3.1. Effects of CRE-SD on osteogenic induction

Many research groups are currently attempting to identify molecules that stimulate osteoblast differentiation for the development of drugs to treat osteoporosis. Plant-derived natural compounds or phytochemicals including flavonoids, polyphenols, lignans, coumarins, terpenoids, carotenoids, and alkaloids have recently been found to stimulate *in vitro* osteoblast cellular differentiation and *in vivo* bone mass formation (Woo, Yonezawa, & Nagai, 2010). Phytochemicals may differentiate mesenchymal stem cells and MC3T3-E1 cells to osteoblasts through several crucial molecular and cellular processes in bone formation and can be used as agents to treat various bone diseases (Woo, Yonezawa, & Nagai, 2010). In this study, we attempted to clarify the effect of CRE-SD, a high water soluble curcuminoids-rich extract, one of plant-derived natural compound or phytochemical, on *in vitro* osteogenic induction and the associated mechanisms, employing MC3T3-E1 cells. Undifferentiated cells, i.e., MC3T3-E1 and C3H10T1/2 were model cell lines utilized for *in vitro* studies on osteoblast differentiation. MC3T3-E1 cells, which were already committed to a specific differentiation phenomenon, can be induced to express osteoblast markers, but these cells had to be reprogrammed by adding epigenetic modifiers (Muhammad et al., 2010). MC3T3-E1 cells can also differentiate into chondrocytes, adipocytes and myoblasts by physiological inducers through Bmp, Wnt signaling circuits (Kobayashi, Maeba, & Takahashi, 2008). CRE-SD induced dose and time dependent activity on cell proliferation and viability of MC3T3-E1 cells upon treatment with different concentrations of CRE-SD (0, 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, and 100 μg/mL). Viable cell numbers were

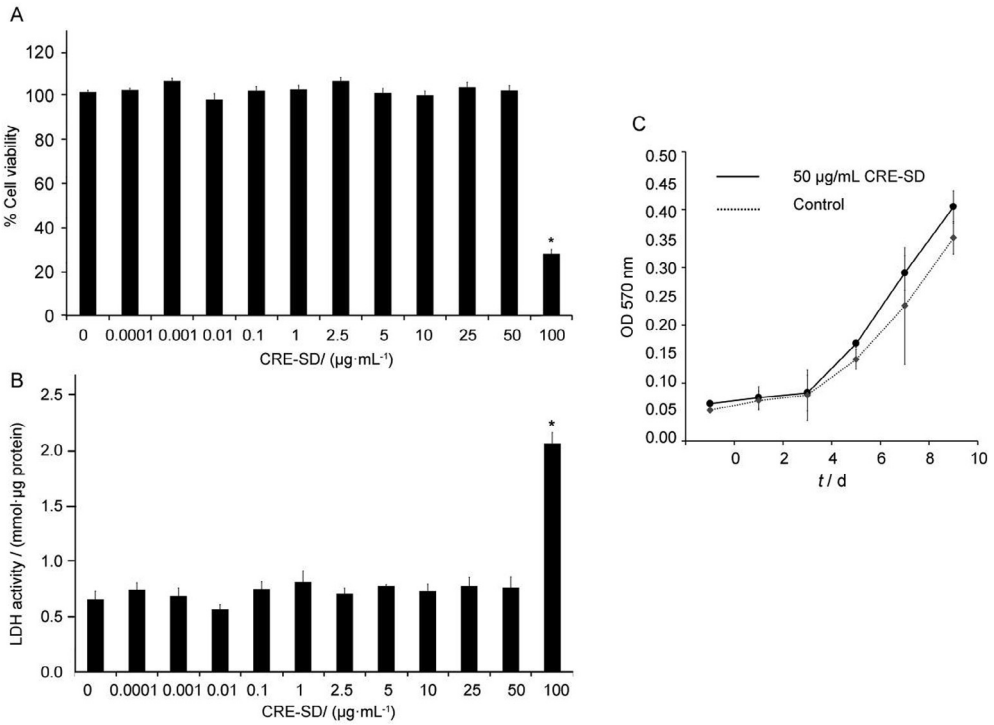

Fig. 1. Cell viability profile and cytotoxicity of CRE-SD. MTT assay for cell viability (A) LDH for cell cytotoxicity (B) and cell proliferation (C). The data represent mean ± SD of three experiments. *P < 0.05 *vs* control cells.

increased up to a 50 μg/mL treatment of CRE-SD and then the higher concentration showed a significant decrease in cell population (Fig. 1A). This indicated the nature of cellular toxicity of CRE-SD. In addition, LDH cell cytotoxicity assay proved beyond that 100 μg/mL of CRE-SD was toxic to the cells (Fig. 1B). In order to discover the fate of CRE-SD on cell proliferation, we conducted MTT based cell proliferation on different days and found that cells number gradually increased up to 8 d and remained constant thereafter at a concentration of 50 μg/mL CRE-SD (Fig. 1C). Several natural compounds have been reported to enhance the alkaline phosphatase (ALP) (an early phase marker of osteoblast differentiation) activity and mineralization (late phase marker) during initial osteogenesis process (Chen et al., 2005; Kobayashi et al., 2008). In this study, ALP activity and mineralization were evaluated to assess the effect of CRE-SD on osteogenic induction. CRE-SD (50 μg/mL) significantly increased ALP activity of MC3T3-E1 cells (Fig. 2A) and C3H10T1/2 cells (Fig. 2B) in a concentration dependent manner. The effect of CRE-SD on two different distinct cell types clearly implies that it is not cell type specific. Cells treated with CRE-SD for 3 weeks showed significant deposits of calcium indicating stimulation of mineralization (Fig. 3). It has been reported that the methoxy substituent in polyphenol derivatives is important to elicit osteogenic activity (Kobayashi et al., 2008). Therefore, the methoxy groups of curcuminoids, may play an important role in inducing initial osteogenic activity. Results thus far, strengthen the concept that CRE-SD stimulated the process of osteoblast induction through an increase in ALP production at the initial stage, and mineralization at the later stage.

Next, the effects of CRE-SD on expression of osteoblast marker genes, namely Bmp-2, Runx2, Collagen 1a and Osteopontin were evaluated. BMP signaling pathway is crucial for early progression and maturation of osteogenesis (Nohe et al., 2002; Chen, Zhao, & Mundy, 2004; Lee, Shin, Min, & Kim, 2008). Bmp-2, the protein in BMP signaling pathway, is crucial for proliferation and differentia-

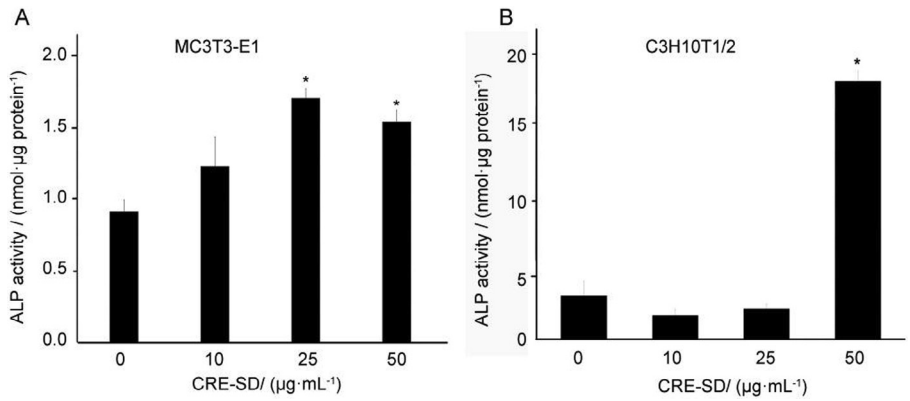

Fig. 2. CRE-SD increased the ALP activity in MC3T3-E1 cells (A) and non-osteogenic mouse pluripotent cell line, C3H10T1/2 cells (B). Values on *Y*-axis represents the nmol ALP production (nmol/μg protein). The data represent mean ± SD of three experiments. *P < 0.05 *vs* control cells.

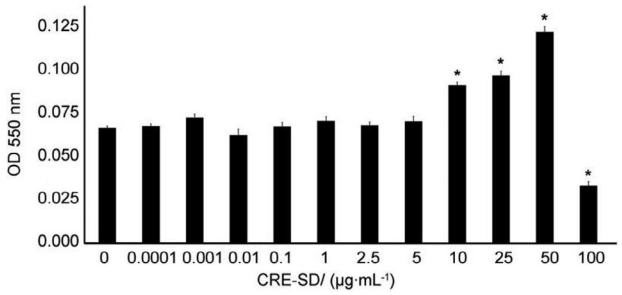

Fig. 3. Effect of CRE-SD on osteogenic differentiation and mineralization in MC3T3-E1 cells. The data represent mean ± SD of three experiments. *P < 0.05 *vs* control cells.

tion of osteogenesis through pre-osteoblast cells, which could depend on the transcription factor Osterix acting downstream of Runx2 (Seib et al., 2009). Collagen 1a and Osteopontin are also known as essential proteins for osteoblast differentiation. Results revealed a dose dependent induction of Bmp-2, Runx2, and Collagen 1a at the RNA level (Fig. 4A), as well as the protein levels (Fig. 4B). The most significant effect was observed in Bmp-2, where 50 μg/ml CRE-SD caused a 3-fold increase in mRNA expression compared with the control and Runx2 protein level (>2-fold increase). On the other hand, CRE-SD did not show any stimulatory effect on Osteopontin either at the gene or protein level. These results collectively indicate that CRE-SD targets upstream molecules like Bmp-2 in osteoblastogenesis cascade.

3.2. Involvement of signaling pathways

The Wnt family plays important roles in many aspects of osteogenesis (Baron & Kneissel, 2013). In addition, canonical Wnt/β-catenin signaling is active in various osteoblast or pre-osteoblastic cell lines such as MC3T3-E1 and cooperatively controls the osteoblast differentiation and bone formation via crosstalk with the BMP signaling pathway, suggesting that the Wnt/β-catenin signaling pathway is an upstream activator of Bmp-2 expression in osteoblasts (Kobayashi et al., 2008). The present study elucidated the possible participation of Wnt/ β-catenin signaling pathway in the effects of CRE-SD on MC3T3-E1 cells differentiation using β-catenin Wnt. CRE-SD (50 μg/mL) increased β-catenin Wnt protein activity significantly (Fig. 5A). In order to study the involvement of BMP signaling pathway on the early stage and Wnt signaling pathway on the late stage with the activity of CRE-SD, we employed inhibitor noggin to inhibit Bmp-2 and Runx2 protein expression to confirm the involvement of CRE-SD on the BMP signaling pathway (Fig. 5B) and employed siRNA mediated silencing technique to knockdown β-catenin Wnt. Silencing of β-catenin Wnt inhibited Bmp-2 protein expression highlighting that the effect of CRE-SD on Bmp-2 was indeed Wnt-dependent (Fig. 5C). It was previously reported that the Wnt/β-catenin

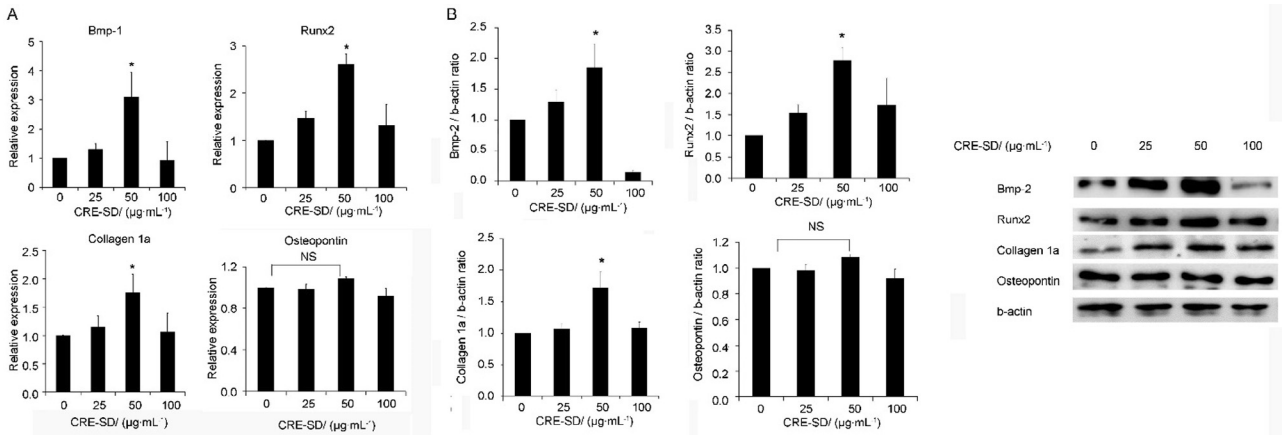

Fig. 4. Effect of CRE-SD on osteogenic marker genes and proteins. The relative mRNA expression levels of the Bmp-2, Runx2, Collagen 1a and Osteopontin against GAPDH were calculated (A). Relative protein expressions of Bmp-2, Runx2, Collagen 1a and Osteopontin were assessed by WB analysis (B) β-actin was used as the housekeeping control. The data represent mean ± SD of triplicate determinations. *P < 0.05 *vs* control cells.

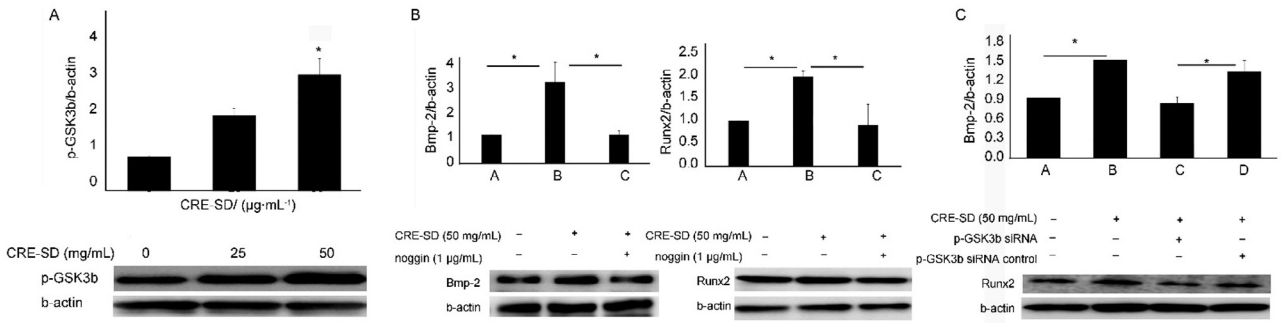

Fig. 5. Effect of CRE-SD in MC3T3-E1 cells on Wnt signaling pathway (A); The influence of CRE-SD in MC3T3-E1 cells was annulled in the presence of Bmp-2 antagonist noggin (B); Effect of Wnt/β-catenin protein silencing on activity of CRE-SD in MC3T3-E1 cells (C). β-actin was used as the housekeeping control. The data represent mean ± SD of triplicate determinations. *P < 0.05 *vs* control cells.

signaling pathway can induce osteoblast differentiation genes, such as ALP, and Collagen 1a (Lum & Beachy, 2004), demonstrating that those genes are up-regulated following cell exposure to CRE-SD. These results suggest that the enhancement of osteoblast differentiation by CRE-SD is likely to correlate with the Wnt/β-catenin signaling pathway. Molecular studies have shown that functional communication between the BMP and Wnt signaling pathways involves multiple mechanisms.

MAPK family also regulates multiple cellular activities related to osteoblast initiation process and can be activated in response to a wide range of external stimuli including natural compounds (MacDonald, Tamai, & He, 2009). Various reports highlight that the MAPK pathway can phosphorylate Runx2 and osterix, implying that MAPK is an obligatory transducer for bone healing (Tian, Xu, Fu, & He, 2011). In addition, MAPK family proteins, p38 and JNK/SAPK, are reported to regulate osteoblast differentiation (Kobayashi et al., 2008). To clarify the possible participation of BMP, p38, JNK/SAPK and Wnt/β-catenin signaling pathways in the effects of CRE-SD on osteoblast differentiation, MC3T3-E1 cells were cultured with CRE-SD in the presence of BMP, p38, JNK/SAPK and Wnt/β-catenin signaling pathways inhibitors, and the ALP activity was measured after 10 d. Only the BMP antagonist noggin and β-catenin siRNA can abolish the CRE-SD promoted ALP activity (Fig. 6). These findings indicate that the osteogenic actions of CRE-SD are mediated by BMP and Wnt/β-catenin signaling pathways.

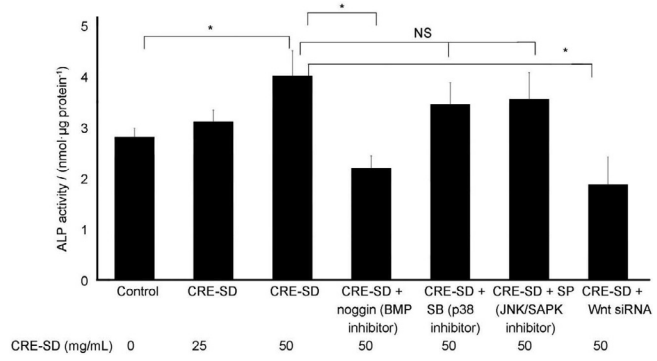

Fig. 6. Rate of ALP production in the presence of signal pathway inhibitors, Bmp-2 antagonist noggin, p38 inhibitor SB, JNK/SAPK inhibitor SP or Wnt/β-catenin protein siRNA. Data represent mean ± SD of triplicate determinations. *$P < 0.05$ *vs* control cells.

4. Conclusion

The present investigation revealed that CRE-SD increases the osteogenic differentiation of MC3T3-E1 cells and non-osteogenic mouse pluripotent cell line, C3H10T1/2. First, the cells become to pre-osteoblast cells as evidenced by over expression of transcription factor Runx2 through BMP signaling pathway. Later, transcription factors Runx2 induce the pre-osteoblast cells to mature osteoblasts. These processes are mediated by Wnt/β-catenin signaling pathway. Taken together, these results indicate that CRE-SD differentiates MC3T3-E1 cells into osteoblasts through the regulation of the Wnt/β-catenin dependent BMP signaling pathway.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Thailand Research Fund (grant number RDG6150075) and Prince of Songkla University (grant numbers MET611036S and MET6202025S). The authors acknowledge Ms. Maria Mullet for help with English language editing.

References

Anand, P., Thomas, S. G., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Sung, B., et al. (2008). Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. *Biochemical Pharmacology, 76*(11), 1590–1611.

Baron, R., & Kneissel, M. (2013). WNT signaling in bone homeostasis and disease: From human mutations to treatment. *Nature Medicine, 19*, 179–192.

Bharti, A. C., Takada, Y., & Aggarwal, B. B. (2004). Curcumin (diferuloylmethane) inhibits receptor activator of NF-κB ligand-induced NF-κB activation in osteoclast precursors and suppresses osteoclastogenesis. *Journal of Immunology, 172*(10), 5940–5947.

Chen, D. i., Zhao, M., & Mundy, G. R. (2004). Bone morphogenetic proteins. *Growth Factors, 22*(4), 233–241.

Chen, K. M., Ge, B. F., Ma, H. P., Liu, X. Y., Ba, M. H., & Wang, Y. (2005). Icariin, a flavonoid from the herb *Epimedium* enhances the osteogenic differentiation of rat primary bone marrow stromal cells. *Pharmazie, 60*, 939–942.

Cheng, A. L., Hsu, C. H., Lin, J. K., Hsu, M. M., Ho, Y. F., Shen, T. S., et al. (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Research, 21*, 2895–2900.

Deschaseaux, F., Sensébé, L., & Heymann, D. (2009). Mechanisms of bone repair and regeneration. *Trends in Molecular Medicine, 15*(9), 417–429.

Feldbrin, Z., Luckish, A., & Shargorodsky, M. (2016). Effects of long-term risedronate treatment on serum ferritin levels in postmenopausal women with osteoporosis: The impact of cardiovascular risk factor load. *Menopause, 45*, 134–141.

Folwarczna, J., Zych, M., & Trzeciak, H. I. (2010). Effects of curcumin on the skeletal system in rats. *Pharmacological Reports, 62*(5), 900–909.

Ireson, C. R., Jones, D. J., Orr, S., Coughtrie, M. W., Boocock, D. J., Williams, M. L., et al. (2002). Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. *Cancer Epidemiology, Biomarkers & Prevention, 11*, 105–111.

Kaewnopparat, N., Kaewnopparat, S., Jangwang, A., Maneenaun, D., Chuchome, T., & Panichayupakaranant, P. (2009). Increased solubility, dissolution and physicochemical studies of curcumin-polyvinylpyrrolidone K-30 solid dispersions. *World Academy of Science, Engineering and Technology, 55*, 229–234.

Kim, W. K., Ke, K., Sul, O. J., Kim, H. J., Kim, S. H., Lee, M. H., et al. (2011). Curcumin protects against ovariectomy-induced bone loss and decreases osteoclastogenesis. *Journal of Cellular Biochemistry, 112*(11), 3159–3166.

Kobayashi, Y., Maeda, K., & Takahashi, N. (2008). Roles of Wnt signaling in bone formation and resorption. *Japanese Dental Science Review, 44*(1), 76–82.

Lao, C. D., Ruffin, M. T., Normolle, D., Heath, D. D., Murray, S. I., Bailey, J. M., et al. (2006). Dose escalation of a curcuminoid formulation. *BMC Complementary and Alternative Medicine, 6*(1). https://doi.org/10.1186/1472-6882-6-10.

Lateh, L., Kaewnopparat, N., & Panichayupakaranant, P. (2018). A method for increasing the water-solubility of curcuminoids and its products. Petty Patent, No. 14639, Thailand.

Lateh, L., Yuenyongsawad, S., Chen, H., & Panichayupakaranant, P. (2019). A green method for preparation of curcuminoid-rich *Curcuma longa* extract and evaluation of its anticancer activity. *Pharmacognosy Magazine, 15*(65), 730–735.

Lee, S. U., Shin, H. K., Min, Y. K., & Kim, S. H. (2008). Emodin accelerates osteoblast differentiation through phosphatidylinositol 3-kinase activation and bone morphogenetic protein-2 gene expression. *International Immunopharmacology, 8*(5), 741–747.

Lum, L., & Beachy, P. A. (2004). The hedgehog response network: Sensors, switches, and routers. *Science, 304*, 1755–1759.

MacDonald, B. T., Tamai, K., & He, X. (2009). Wnt/beta-catenin signaling: Components, mechanisms, and diseases. *Developmental Cell, 17*, 9–26.

Martins, C. A., Leyhausen, G., Volk, J., & Geurtsen, W. (2015). Curcumin in combination with piperine suppresses osteoclastogenesis *in vitro*. *Journal of Endodontics, 41*, 1638–1645.

Mizutani, K., Ikeda, K., Kawai, Y., & Yamori, Y. (1998). Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochemical and Biophysical Research Communications, 253*(3), 859–863.

Muhammad, D., Sha, H. A., Sahidan, S., Mohamad, A., & Rohaya, M. (2010). Determination of the differentiation capacities of murines' primary mononucleated cells and MC3T3-E1 cells. *Cancer Cell International, 10*, 1–12.

Nohe, A., Hassel, S., Ehrlich, M., Neubauer, F., Sebald, W., Henis, Y. I., et al. (2002). The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *Journal of Biological Chemistry, 277*(7), 5330–5338.

Okonogi, S., Oguchi, T., Yonemochi, E., Puttipipatkhachorn, S., & Yamamoto, K. (1997). Improved dissolution of ofloxacin via solid dispersion. *International Journal of Pharmaceutics, 156*(2), 175–180.

Pengjam, Y., Madhyastha, H., Madhyastha, R., Yamaguchi, Y., Nakajima, Y., & Maruyama, M. (2016). Anthraquinone glycoside aloin induce osteogenic initiation of MC3T3-E1 cells: Involvement of MAPK mediated Wnt and Bmp signaling. *Biomolecules & Therapeutics, 24*, 123–131.

Romas, E. (2005). Bone loss in inflammatory arthritis: Mechanisms and therapeutic approaches with bisphosphonates. *Best Practice & Research Clinical Rheumatology, 19*(6), 1065–1079.

Seib, F. P., Franke, M., Jing, D., Werner, C., & Bornhäuser, M. (2009). Endogenous bone morphogenetic proteins in human bone marrow-derived multipotent mesenchymal stromal cells. *European Journal of Cell Biology, 88*(5), 257–271.

Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R., & Srinivas, P. (1998). Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Medica, 64*(04), 353–356.

Sugimoto, E., & Yamaguchi, M. (2000). Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. *International Journal of Molecular Medicine, 5*, 515–520.

Tian, Y. e., Xu, Y., Fu, Q., & He, M. (2011). Parathyroid hormone regulates osteoblast differentiation in a Wnt/β-catenin-dependent manner. *Molecular and Cellular Biochemistry, 355*(1-2), 211–216.

Vali, B., Rao, L., & Elsohemy, A. (2007). Epigallocatechin-3-gallate increases the formation of mineralized bone nodules by human osteoblast-like cells. *Journal of Nutritional Biochemistry, 18*(5), 341–347.

Wang, Z., Sugano, E., Isago, H., Hiroi, T., Tamai, M., & Tomita, H. (2011). Differentiation of neuronal cells NIH/3T3 fibroblast under defined conditions. *Development Growth & Differentiation, 53*, 357–365.

Woo, J. H., Kim, Y. H., Choi, Y. J., Kim, D. G., Lee, K. S., Bae, J. H., et al. (2003). Molecular mechanisms of curcumin-induced cytotoxicity: Induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. *Carcinogenesis, 24*(7), 1199–1208.

Woo, J. T., Yonezawa, T., & Nagai, K. (2010). Phytochemical that stimulate osteoblastic differentiation and bone formation. *Journal of Oral Biosciences, 52*, 15–21.

Yonezawa, T., Lee, J. W., Hibino, A., Asai, M., Hojo, H., Cha, B. Y., et al. (2011). Harmine promotes osteoblast differentiation through bone morphogenetic protein signaling. *Biochemical and Biophysical Research Communications, 409*(2), 260–265.