Original Article

Osteogenic differentiation potential of canine bone marrow-derived mesenchymal stem cells under different β-glycerophosphate concentrations *in vitro*

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Abstract

Stem cell-based therapy employing bone marrow-derived mesenchymal stem cells has been proposed as a promising approach for bone regenerative treatment. Despite numerous studies supporting the application in human medicine, such information in veterinary practice is still lacking. Thus, this study aimed to investigate *in vitro* osteogenic differentiation potential of canine bone marrow-derived mesenchymal stem cells under different doses of β-glycerophosphate supplement. The isolated cells showed self-renewing ability and expressed pluripotent marker genes, *zinc finger protein 42* and *octamer-binding transcription factor 4*, suggesting their potentiality *in vitro*. *In vitro* osteogenic differentiation was performed by using a regular osteogenic induction medium containing 10 mM β-glycerophosphate while 20 mM and 40 mM doses of β-glycerophosphate were used as treatment intervention. At day 14 post-induction, results showed that the levels of increased alkaline phosphatase activity and matrix mineralization upon induction were comparable among the three osteogenic groups. For osteogenic gene marker expression, the β-glycerophosphate supplement showed an upregulating trend of gene set related to osteoblastic differentiation in a dose-dependent manner including *osterix*, *collagen type I alpha 1*, and *osteocalcin*. The 20 mM and 40 mM β-glycerophosphate supplements showed a suppressing trend of an osteochondrogenic marker, *runt-related transcription factor 2*, while an upregulating trend of gene encoding mineralization-inhibiting protein, *osteopontin*, was found in all doses of β-glycerophosphate supplement. In conclusion, β-glycerophosphate benefits *in vitro* osteogenic induction of cBM-MSCs. However, a controversial effect in mineralization was found. Further studies aiming at enhancing alkaline phosphatase activity will benefit osteogenic induction in terms of matrix mineralization.

Keywords: β-glycerophosphate, canine bone marrow-derived mesenchymal stem cells, osteogenic differentiation

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Introduction

Stem cell (SC)-based therapy aiming for bone regeneration has been wildly studied since the past decades. Such research employed laboratory animal models for representing acquired or induced human bone defects (Seebach et al., 2012; Gao et al., 2013a; Sandor et al., 2013). The studies showed promising outcomes suggesting the trend of SC application for bone regeneration and bone tissue engineering *in vitro* and *in vivo*. Among the SC resources, bone marrow-derived mesenchymal stem cells (BM-MSCs) have been introduced as a potential candidate for applying in autologous and allogenic bone regenerative therapy (Palumbo and Li, 2013; Wu et al., 2016). BM-MSCs isolated from human and laboratory animals showed osteogenic differentiation capability *in vitro* and could regenerate bone defects in induced animal models (Meng et al., 2016). Immunomodulatory property and culture expandability have been considered as beneficial properties supporting clinical use of the cells (Hoornaert et al., 2016; Wu et al., 2016).

Despite numerous supporting studies of BM-MSC application in human bone regenerative treatment, information regarding such issue in veterinary practice is still lacking (Chung et al., 2012; Spencer et al., 2012). In this regard, canine SCs have been considered as a pioneer model due to importance in clinical practice (Jung et al., 2009; Chung et al., 2012). Isolation and *in vitro* osteogenic differentiation of canine BM-MSCs (cBM-MSCs) have been reported (Spencer et al., 2012; Csaki et al., 2007). Most of the studies employed regular osteogenic induction protocol. Only a few publications suggested modified techniques to enhance differentiation potency. Recently, Chung et al. (2012) have reported the influence of hypoxic condition on osteogenic differentiation by cBM-MSC. The study showed that hypoxia, by reduction in oxygen tension, hampered osteogenic differentiation potential of the cells in terms of mRNA expression and matrix mineralization. The mRNA expressions of *osterix (Osx)*, *runt-related transcription factor 2 (Runx2)*, *osteocalcin (Ocn)*, and *collagen type I alpha 1 (Col I A 1)* were down regulated upon the osteogenic induction in 1% and 5% oxygen tension compared with 21%.

In terms of osteogenic induction by human MSCs (hMSCs), various techniques have been used to enhance osteogenic differentiation potential of the cells. Employment of osteogenic-inducing molecules and osteogenic-enhancing protein/transcription factors has been reported (Gao et al., 2013b; Gao et al., 2013c; Li et al., 2013; Monteiro et al., 2014). Besides, osteogenic-related effects of some ordinary chemicals have been reported. Phosphate, a simple mineral, is essential for osteogenesis *in vitro* and *in vivo*. Optimized concentration of phosphate has been reported as an *in vitro* osteoinductive molecule in both 2-dimension (D) and 3-D osteogenesis models (An et al., 2015; Zhang et al., 2015). Several phosphate-incorporated 3-D scaffold types aiming for osteo- and mineralo-induction have been developed, e.g. keratin-polycaprolactone (PCL)-hydroxyapatite (HA) based scaffold and poly (lactic-co-glycolic acid) (PLGA) microfibrous scaffold (Zhang et al., 2015; Zhao et al., 2015). In 2-D osteogenesis model, an optimized concentration of β-glycerophosphate was supplemented in an osteogenic induction medium, serving as a phosphate donor. It seems that an optimized concentration of phosphate donor for osteo-induction of hMSCs has been well established. However, such information for osteogenesis by cBM-MSCs is still unclear. Thus, this study aimed to investigate the effect of β-glycerophosphate supplement on osteogenic differentiation by cBM-MSCs *in vitro*.

Materials and Methods

cBM-MSCs isolation and expansion: The protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol #1531038). cBM-MSCs were isolated from bone marrow aspirate according to a protocol modified from previous published reports (Chung et al., 2012; Screven et al., 2014). In summary, 15-20 mL of bone marrow was collected from the iliac crest using 18-guage Jamshidi® bone marrow biopsy aspiration needles (BD, USA). The bone marrow was aspirated into a sterile plastic syringe containing heparin in a ratio of 5,000 IU heparin to 2 mL bone marrow aspirate. With aseptic technique, the bone marrow aspirate was then transferred to a 50 mL sterile conical tube and processed to harvest cBM-MSCs. The cells were washed by adding equal volume of Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific Corporation, USA), then gently mixed and centrifuged at 300 g for 15 minutes. Pellet was resuspended and washed again with 20 mL HBSS, then centrifuged at 1,000 g for 5 minutes. After that, the pellet was resuspended with a standard culture medium and seeded into T-75 culture flasks (Corning, USA). High glucose-Dulbecco's Modified Eagle Medium (HG-DMEM) (Thermo Fisher Scientific Corporation, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Corporation, USA), 1% antibiotic-antimycotic (Thermo Fisher Scientific Corporation, USA), and L-glutamine (Thermo Fisher Scientific Corporation, USA) was used as standard culture medium. The cells were maintained at 37°c, in a humidified atmosphere with 5% CO_2 aeration. After incubation for 48 hours, the cultures were gently washed with HBSS to remove non-adherent cells. The culture medium was routinely substituted every 48 hours. At 90% confluence, the cells were trypsinized and subcultured using 0.25% trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific Corporation, USA). The cells in passage 2-5 were used in the study.

Osteogenic differentiation of cBM-MSCs: An *in vitro* osteogenic induction was performed according to our published protocol (Sawangmake et al., 2014a; Sawangmake et al., 2014b). Briefly, cells were seeded into a 24-well culture plate at a concentration of 2.5 x 10^4 cells/well. After 24 hours, the cells were maintained in an osteogenic induction medium for 14 days with routine 48-hour substitution. The osteogenic induction medium was the standard culture medium

supplemented with 50 mg/mL ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate. To explore an effect of phosphate supplement on osteogenic differentiation, the osteogenic induction medium containing 10 mM β-glycerophosphate was considered as regular induction medium (β-Pi 1X) while the induction medium containing 20 mM (β-Pi 2X) and that containing 40 mM (β-Pi 4X) β-glycerophosphate were set as testing intervention.

Alkaline phosphatase activity assay: Alkaline phosphatase activity assay was performed by using *p*-nitrophenol phosphate as the assay substrate. Cells were lysed in alkaline lysis buffer and separated into two aliquots for alkaline phosphatase activity assay and bicinchoninic acid protein determining assay. For alkaline phosphatase activity assay, the aliquot was incubated in solution comprising 2 mg/mL *p*-nitrophenol phosphate (Thermo Fisher Scientific Corporation, USA), 0.1 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich, USA) and 2 mM MgCl2 (Sigma-Aldrich, USA) at 37°c for 30 minutes, then the reaction was stopped by adding 50 mM NaOH. Absorbance of the alkaline phosphatase was measured at 410 nm. Total cellular protein was analyzed according to Pierce™ BCA protein assay kit (Thermo Fisher Scientific Corporation, USA). Alkaline phosphatase enzymatic activity was calculated by normalizing with total cellular protein amount.

Table 1 Primer sequences

Gene	Accession number	Sequences	5′ 3′	Length (bp)	Tm (°C)
Zinc finger protein 42 (ZFP42 or Rex1)	*XM_003639567.1*	Forward	AGGTTCTCACAGCAAGCTCA	199	59.24
		Reverse	CCAGCAAATTCTGCGCACTG		60.73
Octamer-binding transcription factor 4 (Oct4)	*XM_538830.1*	Forward	AGGAGAAGCTGGAGCAAAACC	100	60.55
		Reverse	GTGATCCTCTTCTGCTTCAGGA		59.50
Osterix (Osx)	*XM_844688.3*	Forward	GCGTCCTCCCTGCTTGAG	122	60.13
		Reverse	GCTTTGCCCAGTGTCGTTG		60.01
Runt-related transcription factor 2 (Runx2)	XM_005642335.1	Forward	GGAAGAGGCAAGAGTTTCACC	209	58.84
		Reverse	GTGCTCACTTGCCAACAGAA		58.89
Osteopontin (Opn)	XM_003434024.2	Forward	GCCACAGAGCAAGGAAAACTC	180	59.73
		Reverse	CTGCTTCTGAGATGGGTCAGG		60.13
Osteocalcin (Ocn)	XM_547536.4	Forward	GCCAGCCTATGGTCTCCTCTG	249	61.90
		Reverse	CCACCAGCTCCTTCTGTTCTCT		54.55
Collagen type I alpha 1 (Col I A1)	NM_001003090.1	Forward	CCAGCCGCAAAGAGTCTACAT	150	60.41
		Reverse	CTGTACGCAGGTGACTGGTG		60.67
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	NM_001003142.1	Forward	CCAACTGCTTGGCTCCTCTA	100	59.38
		Reverse	GTCTTCTGGGTGGCAGTGAT		59.67

Alizarin Red S staining: Cells were washed with phosphate-buffered saline (PBS) solution and fixed with 10% buffered formalin for 15 minutes at room temperature. After that, the fixed cells were washed with deionized water and then stained with 0.5% Alizarin Red S solution (Sigma-Aldrich, USA) for 30 minutes at room temperature. Non-stained dye was washed out with deionized water and let dry at room temperature. The stained cells were captured using flatbed scanner.

For quantification of the staining, 10% cetylpyridinium chloride (CPC) (Sigma-Aldrich, USA) diluted in 10 mM sodium phosphate was used to elute the stained dye. Light absorbance of the solution was then measured at 550 nm of wavelength.

Reverse transcription-polymerase chain reaction (qRT-PCR): According to the manufacturers' directions, total cellular RNA was isolated using TRIzol reagent (Thermo Fisher Scientific Corporation, USA) and Direct-zol™ RNA MiniPrep kit (Zymo Research, USA). On-column DNase I treatment was also performed to digest contaminating genomic DNA. Complimentary DNA (cDNA) was obtained by converting 1 µg RNA sample using ImProm-II™ Reverse Transcription System kit (Promega, USA). Oligo (dT) was used as primer to synthesize first-strand cDNA. Reverse transcription (RT) reaction was performed at 42°C for 90 min and 99°C for 2 min. For quantitative real-time PCR (qPCR), mRNA expression was analyzed by using FastStart-Essential DNA Green Master kit (Roche Diagnostics, USA) and LightCycler® 96 Real-Time PCR system (Roche Diagnostics, USA). Primer sequences were designed to span over exon-exon junction to avoid amplification of residual genomic DNA. qPCR reaction was performed at 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec. Gene expression levels were illustrated as relative mRNA expression by normalizing to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* expression and then the control according to the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [Ct^{\text{target gene}} - Ct^{GAPDH}]_{treated} - [Ct^{\text{target gene}} - Ct^{GAPDH}]_{control}$. Ct referred to cycle threshold.

Statistical analysis: Results were shown as mean ± standard deviation (SD) and analyzed by using two-independent Student *t*-test for two sample group comparison. One-way analysis of variance (ANOVA) followed by Dunnett post hoc analysis were used for three or more group comparison. Three subjects (n=3) were used in the study. Statistically significant difference was recognized when p-value < 0.05.

Results

Isolation of cBM-MSCs: cBM-MSCs were isolated from the bone marrow aspirate, and then cultured and

expanded *in vitro*. The cells adhered to the culture plate and showed fibroblast-like morphology (Figs. 1A and B). mRNA expression of stemness markers were examined, and the expressions of *zinc finger protein 42 (ZFN42 or Rex1)* and *octamer-binding transcription factor 4 (Oct4)* were found (Fig. 1C). For stemness marker mRNA analysis, mRNA expression of the target gene normalized to the reference gene, *GAPDH*, was used. During *in vitro* culture, the cells were viable and able to proliferate according to our routine screening protocol, MTT assay (data not shown). The results suggested that cBM-MSCs could be isolated and expanded *in vitro*, and the expression of mRNA markers indicating potentiality of the cells was found.

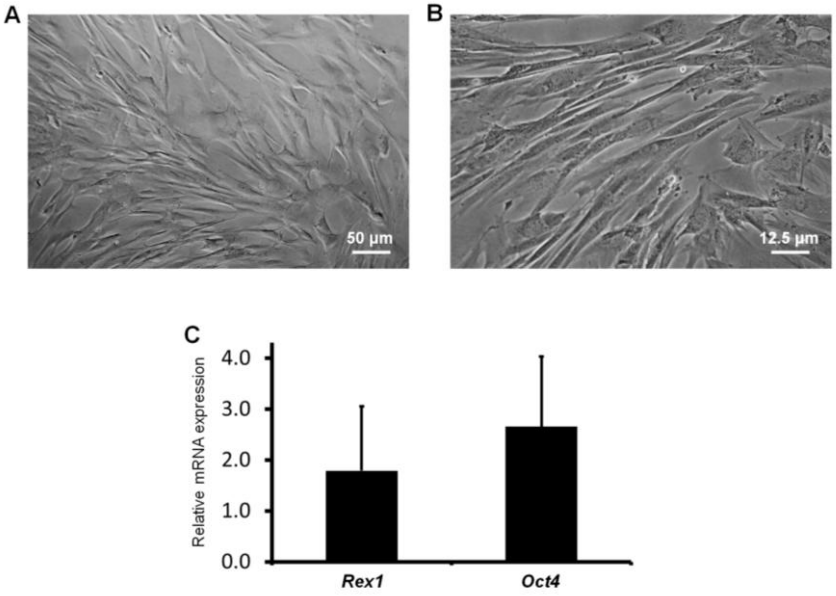

Figure 1 Characterization of isolated canine bone marrow-derived mesenchymal stem cells (cBM-MSCs). Morphological appearances of the isolated cBM-MSCs at 100X (A) and 400X (B) magnifications were observed. mRNA expression of stemness markers, *Rex1* and *Oct4*, was analyzed (C). mRNA expression of the genes was normalized to reference gene, *GAPDH*.

In vitro osteogenic differentiation of cBM-MSCs: In this study, the 14-day osteogenic induction protocol was used. A set of protocols including alkaline phosphatase activity assay, Alizarin Red S staining, and exploration of osteogenic gene expression was used to confirm the differentiation. At day 14 post-induction, the alkaline phosphatase activity of the osteogenic induced cells was significantly higher than that of the undifferentiated control group (Fig. 2A). The detection of matrix mineralization by Alizarin Red S staining showed that the osteogenic group was positively stained with Crimson red color of the dye, suggesting an accumulation of minerals on culture surface (Fig. 2B). qRT-PCR was employed for the analysis of a representative osteogenic gene expression. An upregulation of *Ocn*, a marker indicating mineralization stage, was found upon osteogenic differentiation (Fig. 2C). The results illustrated the osteogenic differentiation potential of cBM-MSCs after the induction *in vitro*.

Effects of β-glycerophosphate supplement on osteogenic differentiation by cBM-MSCs in vitro: To examine whether β-glycerophosphate supplement plays a role in osteogenic differentiation, different concentrations of the phosphate donor, β-glycerophosphate, were added to the osteogenic induction medium. The regular induction medium containing 10 mM β-glycerophosphate was considered as the 1X phosphate supplement (β-Pi 1X) group. The 2X phosphate (β-Pi 2X) and 4X phosphate (β-Pi 4X) groups were employed as the testing intervention. The effects were examined in aspects of alkaline phosphatase activity, matrix mineralization, and osteogenic gene expression. At day 14 post-induction, the alkaline phosphatase activity of all osteogenic induction groups was significantly increased compared with that of the undifferentiated control, but there were no differences in alkaline phosphatase activity among the three phosphate supplemented groups (Fig. 3A). For detection of the matrix mineralization by Alizarin Red S staining, all osteogenic induction groups were positively stained while the undifferentiated control was unstained (Fig. 3B). Quantification of the staining showed that the staining of all osteogenic induction groups was significantly higher than that of the undifferentiated control, but there were no significant differences among the β-Pi 1X, β-Pi 2X, and β-Pi 4X groups (Fig. 3B). The pattern of stained dye in the β-Pi 2X and β-Pi 4X groups was slightly dispersed while the pattern in the β-Pi 1X group was more focalized (Fig. 3B). Regarding osteogenic gene expression, the β-glycerophosphate supplemented groups showed a trend of mRNA upregulation of the osteogenic markers in a dose-dependent manner, including *Osx*, *Ocn*, and *Col I A1* (Fig. 4A). Besides, an increasing trend of *osteopontin (Opn)* mRNA expression was shown, but the pattern was not dose-related (Fig. 4A). For *Runx2* mRNA expression, the regular induction protocol (β-Pi 1X) could enhance a trend of the gene expression, but the β-Pi 2X and β-Pi 4X phosphate supplemented groups could not (Fig. 4A). According to the analysis of osteogenic mRNA expression, due to the variation

of primary cell culture obtained from distinct subjects and the limited number of subjects, alteration in the trends of gene expression was found, but statistical differences were not noted.

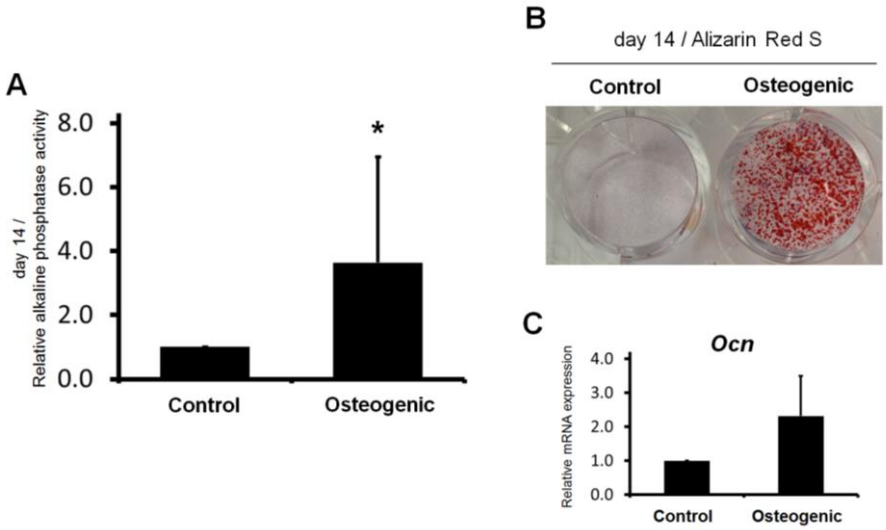

Figure 2 ***In vitro*** **osteogenic differentiation of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs).** *In vitro* osteogenic differentiation potential of cBM-MSCs was confirmed by set of protocols. At day 14 post-induction, alkaline phosphatase activity assay (A) along with Alizarin Red S staining for matrix mineralization (B) were performed. mRNA expression of *osteocalcin (Ocn)*, a marker indicating mature osteoblastic differentiation, was analyzed using qRT-PCR (C). mRNA expression of the gene was normalized to reference gene, *GAPDH*, and the undifferentiated control. The asterisks indicate statistical significance compared with the control.

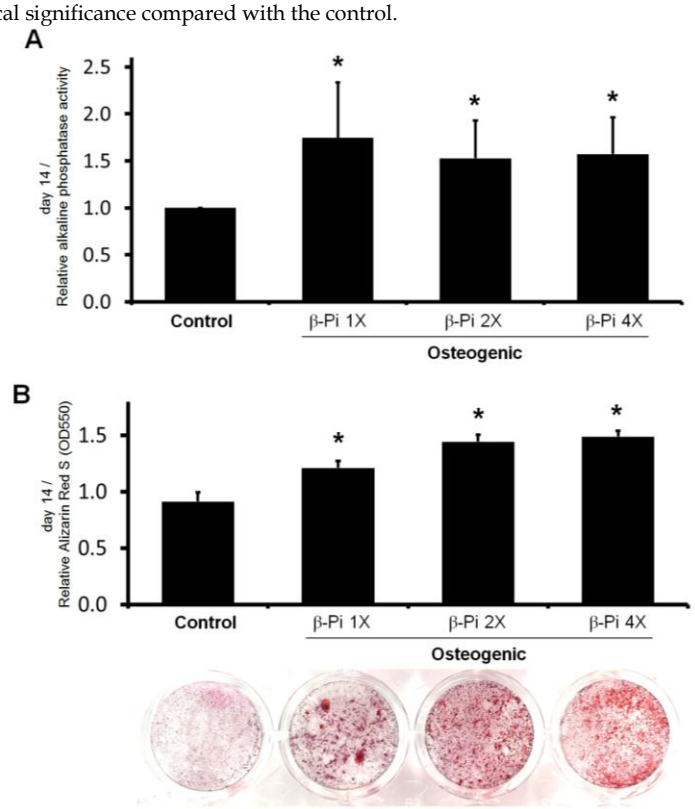

Figure 3 **Effects of β-glycerophosphate supplement on osteogenic differentiation by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) *in vitro*.** The effects of phosphate supplement on *in vitro* osteogenic differentiation potential of cBM-MSCs were explored. The regular osteogenic induction medium containing 10 mM β-glycerophosphate was assigned as 1X phosphate supplement (β-Pi 1X). β-Pi 2X and β-Pi 4X contained 20 mM and 40 mM β-glycerophosphate, respectively. At day 14 post-induction, alkaline phosphatase activity was analyzed (A). Matrix mineralization of differentiated cells was explored and quantified by Alizarin Red S staining (B). The asterisks indicate statistical significance compared with the control.

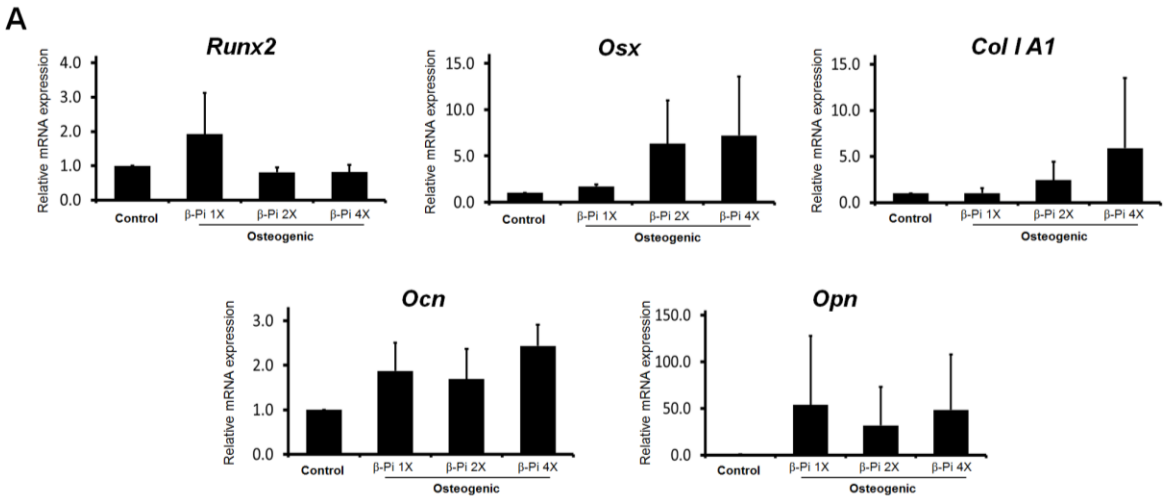

Figure 4 **Effects of β-glycerophosphate supplement on osteogenic differentiation by canine bone marrow-derived mesenchymal stem cells (cBM-MSCs) *in vitro*.** The effects of phosphate supplement on osteogenic gene expression of cBM-MSCs which underwent *in vitro* osteogenic induction were analyzed. At day 14 post-induction, a set of osteogenic gene markers referring osteochondrogenic progenitor (*runt-related transcription factor 2*, *Runx2*), osteoblast progenitor (*osterix*, *Osx*), pre-osteoblast (*collagen I alpha 1*, *Col I A1*), mature osteoblast (*osteocalcin*, *Ocn*), and mineralization inhibitor (*osteopontin*, *Opn*) were analyzed using qRT-PCR. mRNA expressions of the genes were normalized to reference gene, *GAPDH*, and the undifferentiated control.

Discussion

This study showed that cBM-MSCs were efficiently isolated from bone marrow aspirates and could be expanded *in vitro*. The isolation protocol was slightly modified from previous publications (Chung et al., 2012; Screven et al., 2014). According to previous published reports, the cells were attach-dependent and showed fibroblast-like morphology (Chung et al., 2012; Screven et al., 2014). The expression of core stemness mRNA markers, *Rex1* and *Oct4*, along with an *in vitro* self-renewing capacity, could preliminarily confirm their stem cell characteristics.

For *in vitro* osteogenic differentiation potential of cBM-MSCs, the 14-day osteogenic induction protocol according to previous published reports was employed (Sawangmake et al., 2014a; Sawangmake et al., 2014b). This study used a set of protocols to confirm osteogenic differentiation capacity including alkaline phosphatase activity assay, Alizarin Red S staining, and detection of osteogenic mRNA markers expression. This set of protocols was previously published in our studies (Sawangmake et al., 2014a; Sawangmake et al., 2014b). At day 14 post-induction, the osteogenic induction of the cells was preliminarily confirmed by the increase in alkaline phosphatase activity and matrix mineralization. The quantitative alkaline phosphatase activity assay was used in this study and the result showed that approximately 2-3 folds of activity increased. Chung et al. (2012) published a report regarding the osteogenic induction of cBM-MSCs by using a qualitative alkaline phosphatase activity staining assay. The published results showed an unclear staining pattern of alkaline phosphatase activity within the cells. However, together with Alizarin Red S staining, the authors concluded that the cells underwent osteogenic differentiation. For osteogenic gene marker expression, this present study found the upregulation of the representative marker indicating mineralization stage, *Ocn*, upon induction. This could confirm that cBM-MSCs contain osteogenic differentiation potential *in vitro*.

To investigate the effects of β-glycerophosphate supplement on osteogenic differentiation by cBM-MSCs, different concentrations of the phosphate donor, β-glycerophosphate, were used, supplemented in the regular osteogenic induction medium. According to our previous published protocol, the osteogenic induction medium containing 10 mM β-glycerophosphate was considered as 1X supplement (Sawangmake et al., 2014a; Sawangmake et al., 2014b). By adding more concentration of β-glycerophosphate, the 2X and 4X supplement groups were created, which contained 20 mM and 40 mM β-glycerophosphate, respectively. The osteogenic differentiation potential was analyzed in aspects of quantitative alkaline phosphatase activity, matrix mineralization, and set of osteogenic marker mRNA expression. Upon osteogenic induction by regular induction medium, the alkaline phosphatase activity was increased. However, the supplementation of phosphate donor could not enhance a dose-dependent increase in the alkaline phosphatase activity as shown by the comparable level of alkaline phosphatase activity in all osteogenic induction groups. In addition, the degrees of matrix mineralization as detected by Alizarin Red S staining among the three different phosphate supplemented groups were not clearly different. Just a slight distinction in the dye staining pattern in the osteogenic induction groups was noticed. The 20 mM and 40 mM β-glycerophosphate supplements led to the distribution of matrix mineralization as shown by the dispersed staining pattern while the regular induction medium containing 10 mM β-glycerophosphate caused the focalized staining pattern. These results correlate with previous publications suggesting some controversial effects of phosphate donor supplement in osteogenic induction (Beck et al., 2000; Addison et al.,

2007). As β-glycerophosphate serves as an organic phosphate donor, the alkaline phosphatase enzymatic cleavage of the molecule into pyrophosphate (PPi) and phosphate (Pi) is indeed necessitated (Addison et al., 2007). Phosphate is essential for matrix mineralization, but pyrophosphate plays a paradoxical role in inhibiting mineralization (Addison et al., 2007). In addition, β-glycerophosphate is classified as a classical serine-threonine phosphatase inhibitor; very high concentration of β-glycerophosphate might cause alkaline phosphatase activity blockage (Addison et al., 2007). It is suspected that β-glycerophosphate in high concentration might suppress an alkaline phosphatase activity and/or accumulate high extracellular pyrophosphate concentration. In this regard, a study that involves enhancing alkaline phosphatase activity by increasing concentrations of ascorbic acid and dexamethazone in osteogenic induction medium will be beneficial.

In terms of osteogenic gene expression, according to the variation of primary cell culture obtained from distinct subjects and limited number of donor subjects, the alteration in trends of gene expression could be observed. However, the statistical significances were not noted. Regarding the results, this study found that β-glycerophosphate supplement showed the trend of a dose-dependent upregulation of some osteogenic mRNA markers upon induction including *Osx*, *Ocn*, and *Col I A1*. For *Runx2* expression, the regular osteogenic induction medium could enhance a trend of gene upregulation, but this was not found in the 20 mM and 40 mM β-glycerophosphate supplements. It is suggested that β-glycerophosphate supplement might enhance differentiation toward osteoblastic lineage of cBM-MSCs as *OCN* is a marker indicating mature osteoblastic cells (Jones, 2011). Additionally, *Osx* and *Col I A1* correlated with osteoblastic progenitors and pre-osteoblastic cells, respectively (Jones, 2011). Besides, the suppressing effect of high dose of phosphate supplement on *Runx2*, an osteochondrogenic progenitor, might be due to an osteoblastic fate decision of the cells (Jones, 2011). For the trend of *Opn* upregulation, this correlates with previous reports showing an upregulation of the gene upon phosphate supplement during osteogenic induction by murine calvarial osteoblasts, MC3T3-E1 (Beck et al., 2000; Addison et al., 2007). Previous results suggested that *Opn* inhibited matrix mineralization via its negatively charged phosphate residues, which resembled pyrophosphate's mechanism (Addison et al., 2007; Staines et al., 2012). This might be the reason for the controversial results regarding matrix mineralization upon osteogenic induction with high dose of phosphate supplement found in our study.

In conclusion, this study showed that high dose of β-glycerophosphate supplement could benefit *in vitro* osteogenic induction of cBM-MSCs by triggering differentiation fate toward osteoblastic lineage. However, there were some controversial effects regarding matrix mineralization which might be due to the inhibitory effect of β-glycerophosphate on alkaline phosphatase activity and the expression of mineralization inhibitor, *Opn*. Further studies regarding the enhancement of alkaline phosphatase expression and enzymatic activity will benefit the induction protocol in terms of accelerating mineralization of extracellular matrix *in vitro*.

Conflict of interest statement: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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บทคัดย่อ

ความสามารถในการเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกในห้องทดลองของเซลล์ต้นกำเนิดมีเซ็นไคม์จากไขกระดูกสุนัขภายใต้การเสริมด้วยเบตากลีเซอโรฟอสเฟตที่มีความเข้มข้นต่างกัน

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ในการศึกษาแนวทางการรักษาด้วยเซลล์ต้นกำเนิดโดยอาศัยเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกเพื่อใช้ในการฟื้นฟูความเสียหายหรือความผิดปกติของกระดูกนั้น พบว่าเป็นที่ยอมรับในประสิทธิภาพและความเป็นไปได้ของแนวทางการรักษา อย่างไรก็ตามการศึกษาวิจัยส่วนใหญ่มุ่งเน้นการประยุกต์ใช้ในมนุษย์ เป็นผลให้งานวิจัยด้านนี้ในทางสัตวแพทย์ยังมีข้อจำกัด งานวิจัยนี้จึงมุ่งหมายเพื่อศึกษาความสามารถในการเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกในห้องทดลองของเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัขภายใต้การเสริมด้วยเบตากลีเซอโรฟอสเฟตที่มีความเข้มข้นต่างกัน การศึกษาพบว่าเซลล์ต้นกำเนิดที่แยกได้มีความสามารถในการเพิ่มจำนวนในห้องทดลอง และมีการแสดงออกของยีนซิงค์ฟิงเกอร์โปรตีน 42 และอ็อคทาเมอร์บายน์ดิ้งทรานสคริปชันแฟคเตอร์ 4 สำหรับการเหนี่ยวนำให้เปลี่ยนแปลงเป็นเซลล์สร้างกระดูกด้วยอาหารเลี้ยงเซลล์ที่มีส่วนผสมของเบตากลีเซอโรฟอสเฟตที่ความเข้มข้น 10 20 และ 40 ไมโครโมลาร์นั้น พบว่าในวันที่ 14 ภายหลังการเหนี่ยวนำ เซลล์สามารถเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกในห้องทดลอง โดยมีการเพิ่มขึ้นของระดับการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทสและการตกตะกอนแร่ธาตุในห้องปฏิบัติการที่ไม่แตกต่างกัน สำหรับการแสดงออกของยีน พบว่ามีการเพิ่มขึ้นของยีนที่เกี่ยวข้องกับการเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกในระดับที่สัมพันธ์กับความเข้มข้นของฟอสเฟต ได้แก่ ออสเทอริกซ์ คอลลาเจนชนิดที่ 1 และออสทิโอแคลซิน นอกจากนี้ พบว่าการเสริมเบตากลีเซอโรฟอสเฟตที่ระดับ 20 และ 40 ไมโครโมลาร์กดการแสดงออกของยีนรันต์รีเลเทตทรานสคริปชันแฟคเตอร์ 2 ซึ่งเกี่ยวข้องกับการเปลี่ยนแปลงในระยะการแปรสภาพไปสู่เซลล์สร้างกระดูกและเซลล์สร้างกระดูกอ่อน แต่การเสริมเบตากลีเซอโรฟอสเฟตในทุกความเข้มข้นเพิ่มการแสดงออกของยีนออสทิโอพอนทิน ซึ่งมีผลยับยั้งการสะสมของสารอนินทรียในเมทริกซ์ ผลการศึกษานี้แสดงให้เห็นว่าการเสริมฟอสเฟตโดยการใช้เบตากลีเซอโรฟอสเฟตสามารถเพิ่มการเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกของเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัข อย่างไรก็ตาม การตกตะกอนแร่ธาตุในห้องปฏิบัติการที่ไม่เพิ่มขึ้นอาจเป็นผลของเบตากลีเซอโรฟอสเฟตที่ยับยั้งการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทส และการแสดงออกที่เพิ่มขึ้นของออสทิโอพอนทิน การศึกษาเพื่อเหนี่ยวนำการทำงานของเอนไซม์อัลคาไลน์ฟอสฟาเทสอาจช่วยเพิ่มประสิทธิภาพของการเปลี่ยนแปลงเป็นเซลล์สร้างกระดูกในห้องทดลองโดยเพิ่มการตกตะกอนแร่ธาตุในห้องปฏิบัติการ

คำสำคัญ: เบตา-กลีเซอโรฟอสเฟต เซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัข การแปรสภาพเป็นเซลล์สร้างกระดูก

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