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Porcine placenta extract induced Akt, ERK, and JNK signaling to heighten the osteogenic activity of human osteoblasts

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ABSTRACT

Porcine placenta extract (PPE) is an effective pharmaceutical and cosmetic ingredient, despite its high bioavailability. Despite this, only a few studies on the extract's effect on human osteoblast cellular behavior have been conducted. This study used hFOB 1.19 cells to demonstrate the mechanism by which a novel potent osteogenic compound that was discovered by stimulating the growth, alkaline phosphatase secretion, mineralization, and osteogenesis markers of human osteoblast hFOB 1.19 cells resulted in significant increases in extracellular signal-regulated kinase (ERK)1/2, Akt, and c-Jun N-terminal kinase (JNK) phosphorylation. In addition, PPE was discovered to significantly increase *in vitro* bone formation that may provide the beneficial potential for osteoporosis prevention and treatment.

INTRODUCTION

As bones grow and changes throughout a person's lifetime, osteoblasts and osteoclasts work together to continuously and precisely remodel the bones. The most common cause of imbalanced bone remodeling is the deregulated coupling between the major bone cells, the increment of osteoclast resorption activity over osteoblast bone formation rate, or the declination of bone turnover rate. Osteoporosis is a common bone disease in adults and is the consequence of this process (Boyle *et al*., 2003; Rachner *et al*., 2011). New research indicates that long-term use of antiosteoporotic drugs may have negative health effects on humans, despite their ability in order to boost bone strength and reduce the risk of fracture (Curtis *et al*., 2015). It is imperative that new, less toxic agents are developed for osteoporosis prevention and treatment. Numerous drugs have been developed to help prevent and treat this condition. In contrast to the traditional prevention focus on other trace elements such as calcium and vitamin D, the identification of multiple nutrients with bone-sparing properties has been made possible by recent preclinical strategies for prevention. It has been demonstrated that natural products, in particular, can be valuable sources of novel osteoporosis drugs (Schulman *et al*., 2011).

A female mammal's placenta is a temporary organ which grows during pregnancy. The placenta acts as a blood vessel connecting the mother's uterus to the fetus to allow oxygen and nutrients to flow to the developing baby (Kapila and Chaudhry, 2021). As a consequence, the placenta generates and enriches bioactive components such as growth factors and hormones as well as essential minerals, vitamins, and cytokines (Phuapradit *et al*., 2000). Placenta extract is used by a diverse range of mammals, such

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as porcine, bovine, and humans, and has been shown to improve the effectiveness of biological and therapeutic applications such as increasing personal health and energy (Cross, 2016), promoting hair regrowth (Kim *et al*., 2020), reducing and relieving pain (Park and Cho, 2017), and increasing wound healing activity (Shukla *et al*., 2004), along with anti-inflammatory effects (Heo *et al*., 2018), antimicrobial properties (King *et al*., 2007), and antioxidant effects (Rozanova *et al*., 2012). Some studies have shown that porcine placenta extract (PPE) has an enhancement effect on osteoblasts. According to a recent study, the placenta regulates ER stress and the production of reactive oxygen species in osteoblasts (Lee *et al*., 2016), while placental-derived adherent cells prevented bone loss and promoted bone formation by inhibiting the formation of osteoclasts and stimulating the differentiation of osteoblasts in the host (Li *et al*., 2011). Therefore, understanding the mechanism underlying this process would be highly valuable in evaluating the effect of PPE in an in *vitro* assay. The present study demonstrates that PPE can enhance human osteoblast activity, including cell proliferation differentiation and mineralization, mediated by induction of the prosurvival signaling pathway. These results suggest that a possible source of bioingredients from agricultural industry waste for osteoporosis treatment and prevention, according to these findings, is PPE.

MATERIALS AND METHODS

Cell culture

Human osteoblast hFOB 1.19 cells were purchased from the American Type Culture Collection (ATCC). Osteoblast cells were maintained to standard guidelines of good cell culture practice in a 1:1 mixture of Ham's F12 Medium to Dulbecco's Modified Eagle's Medium, with 2.5 mM *L*-glutamine (without phenol red). G418 0.3 mg/ml was added to the base medium, and fetal bovine serum was supplemented at 10% final concentration.

Preparation of PPE

The PPE was obtained from the Faculty of Sciences, Mahidol University, Bangkok, Thailand. The porcine placenta was washed and homogenized in a phosphate buffer saline (PBS) solution. Subsequently, the sonication and centrifugation of the homogenate were conducted at 4°C for 1 hour. Eventually, the supernatant was collected and sterilely filtrated with 0.2 µm filters. The Bradford assay was performed to measure the protein concentration that the PPE lastly labeled with the exact concentration.

Cell viability assessment using MTT assay

Cell viability was determined by the MTT assay. In brief, seeded osteoblast cells were grown overnight at a density of 5×10^3 cells per well in a 96-well cell culture plate. PPE was then added in distinct concentrations and incubated for desired incubation times. At the end of incubation, the MTT reagent was dispensed and sequentially incubated for 4 hours. The Dimethyl sulfoxides (DMSO)-solubilized formazan crystal was used to measure the light absorbance by a microplate spectrophotometer at 570 nm. The cell viability was calculated as follows: [(PPE treated Abs570)/(control Abs570)] × 100 (%).

Alkaline phosphatase activity measurement

Osteoblast cells were grown overnight at a density of 1×10^3 cells per well in a 6-well cell culture plate. The cell culture medium was replaced with an osteogenic induction medium (0.1 µM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/ml of ascorbic acid) containing PPE at the desired concentration and incubated for 3, 5, and 7 days. Treated cells were washed twice with a PBS solution before adding 50 mM Tris-HCl, pH 7.5, plus protease inhibitor cocktail and lysed using ultrasonic sonication. The extract protein was collected using high-speed centrifugation, and enzyme activity was measured by using Cobas c 111 analyzers.

Mineralization assay

Seeded osteoblast cells were grown overnight at a density of 5×10^3 cells per well in a 96-well cell culture plate. The cell culture medium was replaced with an osteogenic induction medium containing PPE at the desired concentration and incubated for 7 and 14 days. Cells were then washed twice with a PBS solution before fixing with 10% formaldehyde for 10 minutes. Subsequently, the cells were stained with Alizarin red and photographed under an inverted microscope to observe mineralization. The level of calcium deposition was quantified by elution of Alizarin red with 10% acetic acid, and the light absorbent was measured at 405 nm. Data is presented as relative to the control (the osteogenic induction medium).

RNA extraction and real-time polymerase chain reaction (PCR)

hFOB 1.19 cells were treated with an osteogenic induction medium and/or with PPE for 4 days. Nucleic acid was extracted with the Total RNA Mini Kit (Bio-Rad, CA) as per the manufacturer's recommendation. First-strand cDNA was synthesized using the Tetro cDNA synthesis kit (Bioline, UK) and real-time PCR was performed. Primer sequences are listed in Table 1. The gene expression was analyzed as the relative quantification with the values of $2^{-\Delta\Delta CT}$, normalized with the GAPDH expression level.

Western blot analysis

Human osteoblast cells were treated with PPE with or without inhibitors at the indicated concentration. The cells were then washed with ice-cold PBS and intracellular protein harvested with a RIPA buffer. Protein concentration was quantified by using the Bradford assay. The protein was then separated by SDS-gel electrophoresis and transferred onto PVDF membranes. After blocking with a 5% skim milk buffer, the membrane was then incubated and gently agitated with anti-pAkt, anti-Akt, anti-pERK, anti-ERK, anti-pJNK, anti-JNK, anti-β-actin, and anti-cyclin D1 (CST, MA) at 4°C overnight. After that, the membranes were washed and incubated with HRP-linked anti-rabbit antibody (CST, MA) for 1 hour at room temperature. The incubated membranes were washed twice and the expression signal was detected by using ChemiDoc™ XRS and analyzed by Image Lab (Bio-Rad, CA).

Statistical analysis

All values are presented as mean ± SEM. One-way analysis of variance was used to determine significant differences between groups, accompanied by the Tukey–Kramer test regarding appropriation. p value < 0.05 was accounted as a statistically significant difference. Statistical testing was analyzed using GraphPad Prism version 7.

Table 1. Primer sequences for quantitative real-time PCR.

Gene name	Primer sequences	Product size (bp)
ALP	F: 5′-CATGGCTTTGGGCAGAAGGA-3′ R: 5′-CTAGCCCCAAAAAGAGTTGCAA-3′	166
COL1	F: 5′-AGCCGCTTCACCTACAGC-3′ R: 5′-TTTTGTATTCAATCACTGTCTTGCC-3′	82
OCN	F: 5′-ATGAGAGCCCTCACACTCCTC-3′ R: 5′-GCCGTAGAAGCGCCGATAGGC-3′	267
RUNX2	F: 5′-CCTAGGCGCATTTCAGATGAT-3′ R: 5′-TAGCGTGCTGCCATTCGA-3′	441
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: 5′-GAAGGTGAAGGTCGGAGTC-3′ R: 5′-GAAGATGGTGATGGGATTTC-3′	266

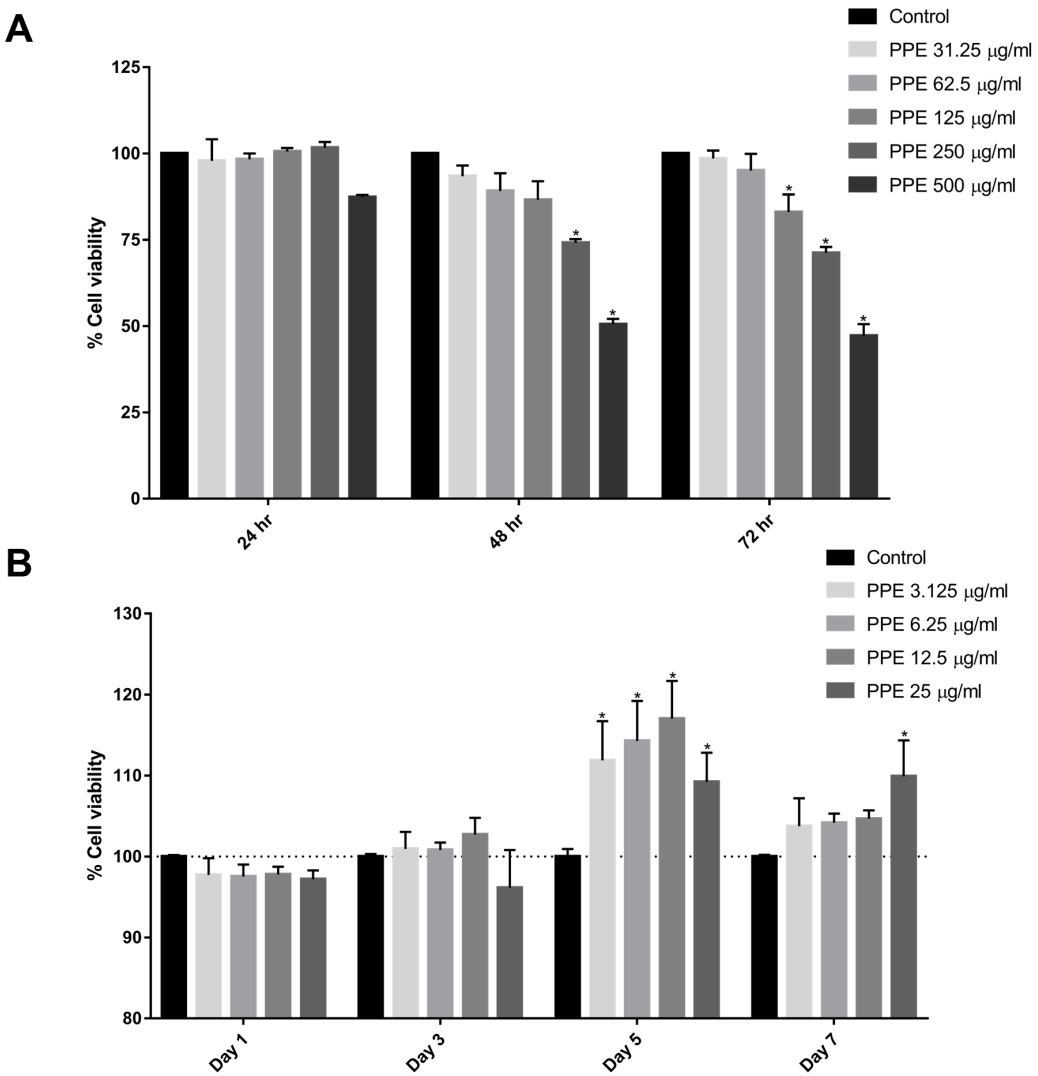

Figure 1. PPE activated osteoblast proliferation. (A) Cytotoxicity screening of PPE on hFOB 1.1.9 cell at 0–500 μg/ml. (B) Proliferation effect of PPE at a concentration of 0–25 μg/ml on human osteoblast cells. hFOB 1.1.9 cells were seeded in a 96-well plate and incubated with the indicated concentration of PPE. Cell viability was accessed using the MTT assay. Data presented as % cell viability normalizing with control. Each value is expressed as mean ± SEM (n = 9). p value < 0.05 was included as a statistically significant difference.

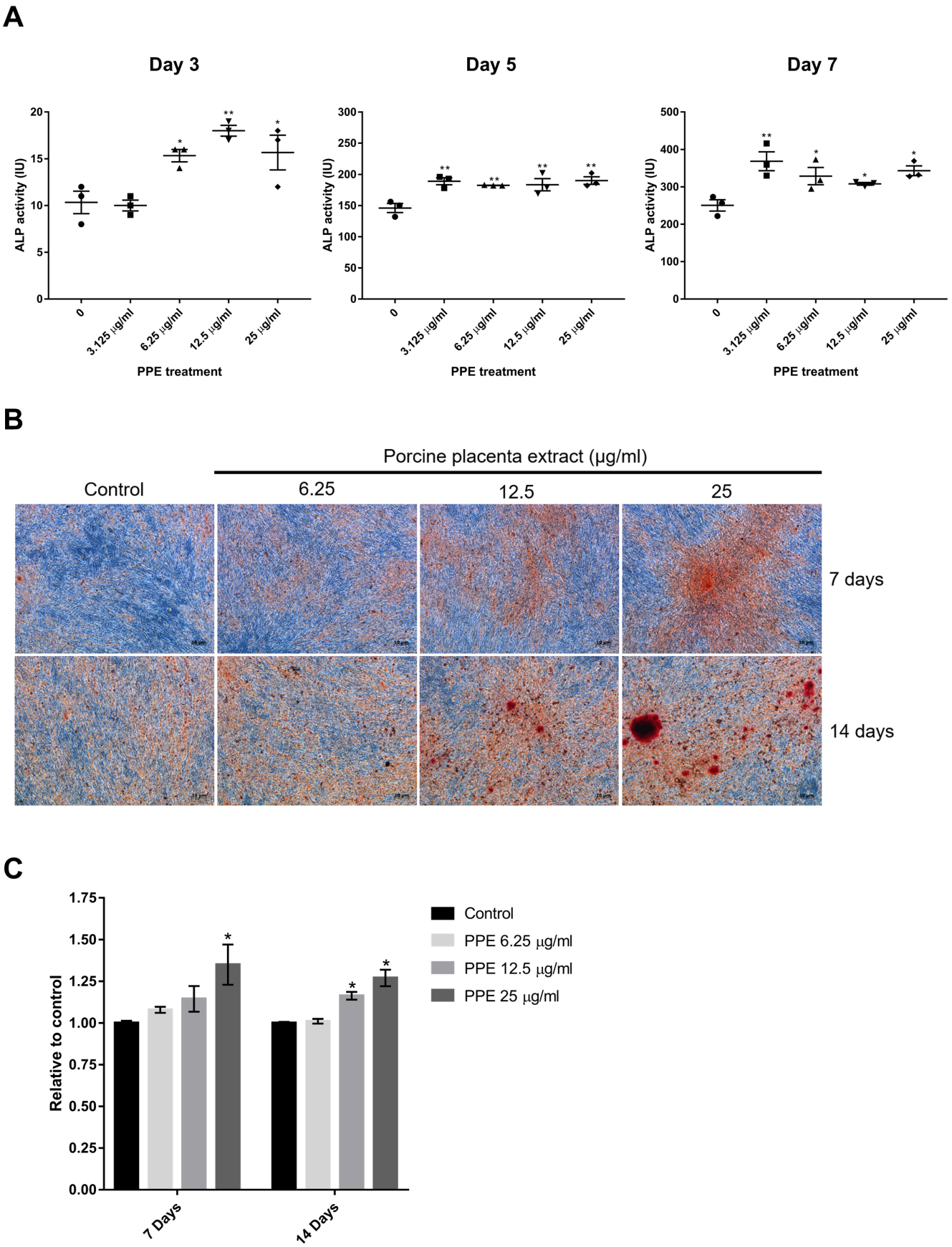

Figure 2. PPE enhances osteogenic phenotypes of human osteoblast cells. hFOB 1.1.9 cell was treated with an osteogenic induction medium supplemented with PPE at the indicated concentration. (A) ALP enzyme activity was measured from total cell lysate at days 3, 5, and 7 and showed as an international unit enzyme. (B) Treated cells were stained with Alizarin red and photographed under an inverted microscope and subsequently eluted for quantification compartment (C). Each value is expressed as mean ± SEM ($n = 6$). p value < 0.05 was included as a statistically significant difference.

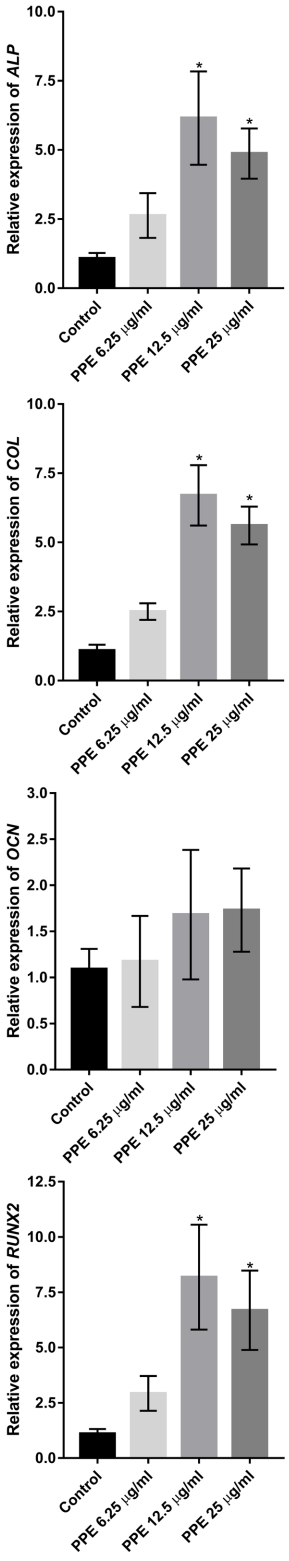

Figure 3. PPE promotes osteogenesis-related genes. hFOB 1.19 cells were seeded into a 6-well plate and subsequently incubated with either osteogenic induction medium or PPE at the indicated concentration for 4 days. The cellular mRNA was extracted and the expression of osteogenesis-related genes measured using real-time PCR. Data was calculated as relative mRNA expression to the osteogenic induction medium group. Data are shown as the mean ± SEM ($n = 6$). p value < 0.05 was included as a statistically significant difference.

RESULTS

PPE-induced osteoblast proliferation

To optimize the nontoxic concentration of PPE, hFOB 1.19 cells were incubated with various concentrations of PPE at 0–500 µg/ml and viability was observed for 3 days. The results show that high concentrations of PPE (over 125 µg/ml) significantly suppressed the viability of osteoblasts. Medium concentrations (62.5–125 µg/ml) slightly suppressed the viability at 48 and 72 hours, whereas low concentrations of PPE did not affect osteoblast viability (Fig. 1A). Therefore, the optimal concentrations of PPE for use in this study were 0–30 µg/ml.

The optimal concentration of PPE was further investigated for osteoblast proliferative effect. After coincubation with PPE for 7 days, 3.125–25 µg/ml of PPE significantly increased cell proliferation rate when compared with the control group (Fig. 1B). The peak of cell proliferation was detected at day 5 regarding PPE concentration, suggesting that a low concentration of PPE could induce osteoblast proliferation.

PPE enhanced osteogenic activity of osteoblast cells

Osteoblast maturation is a crucial process for bone formation, which increases alkaline phosphatase (ALP) enzyme production, matrix deposition, and mineralization. The present study investigated the context of PPE on osteoblast maturation by measuring ALP activity and mineralization. The results show that PPE significantly increased ALP enzyme activity compared with the control, with an incremental effect in a time-dependent manner (Fig. 2A). Moreover, PPE could enhance calcium deposition demonstrated by overstaining of red color when compared with the induction medium group, as shown in Figure 2B. To confirm, stained cells were eluted, and the light absorbent was measured. The results demonstrate that increasing PPE concentration significantly enhanced mineralization in human osteoblasts (Fig. 2C). From these results, it can be concluded that PPE positively enhances osteogenic activity involved with the activation of human osteoblast maturation.

PPE promotes osteogenesis-related markers

To further confirm the osteogenic differentiation of human osteoblasts, PPE-treated cells were measured for their relative expression of osteogenesis-related genes, including *ALP*, *COL1* (collagen type 1), *OCN* (osteocalcin), and *RUNX2* (Runt-related transcription factor 2) genes. PPE treatment markedly increased the expression levels of *ALP*, *COL1*, and *RUNX2* genes; peak expression was observed at 12.5 µg/ml of PPE, whereas the expression level of the *OCN* gene showed an increasing trend compared with the osteogenic induction medium group (Fig. 3). Importantly, PPE promoted osteogenesis differentiation of human osteoblasts.

PPE induced phosphorylation of ERK1/2, Akt, and JNK

The activation of the MAPK, AKT, and JNK signaling pathways is well known to be associated with cellular proliferation and maturation of osteoblast cells. Therefore, the researchers investigated the effects of PPE on the induction of the signaling pathway associated with osteogenesis. The results indicate that

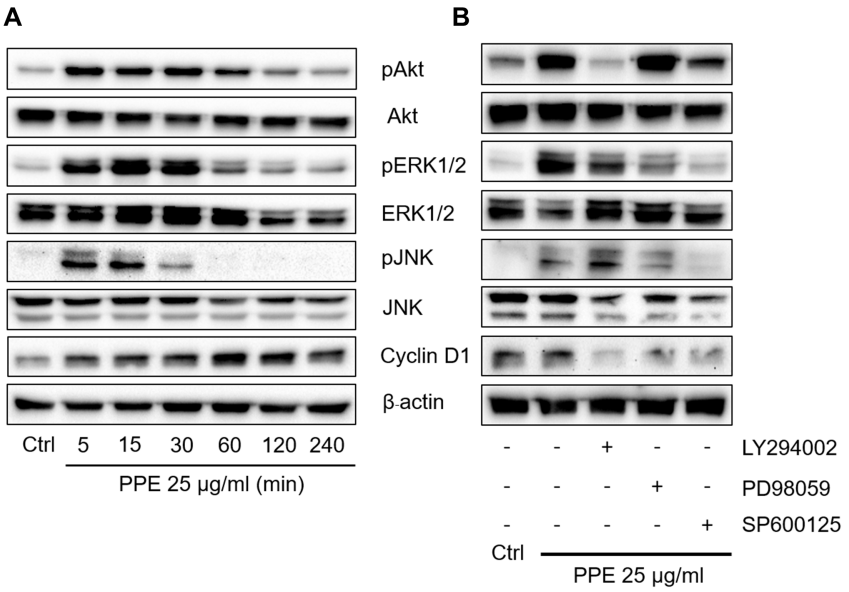

Figure 4. PPE induced phosphorylation of Akt, ERK1/2, and JNK. (A) hFOB 1.19 cells were incubated with and without indicated PPE concentration and times. The survival-associated signaling proteins were determined by using immunoblotting. (B) Additional incubation with LY294002, PD98059, and SP600125 for 15 μM on PPE treatment attenuated signaling protein overexpression. β-actin was used as protein loading control of immunoblotting experiment.

PPE induced phosphorylation of Akt, ERK1/2, and JNK as early as 5–30 minutes after the addition of PPE to the hFOB 1.19 cell lines, respectively (Fig. 4A). Phosphorylation reached a maximum level at 5–60 minutes after exposure and then decreased. These are confirmed in Figure 4B, the addition of LY294002 (Akt inhibitor), PD98059 (ERK inhibitor), and SP600125 (JNK inhibitor) that PPE treatment condition attenuated the phosphorylation of Akt, ERK1/2, and JNK, respectively, when compared with the untreatment condition. These suggest that PPE is involved in the induction of the ERK, PI3K-AKT, and JNK signaling pathways.

The molecular mechanism of PPE was further investigated, targeting cell cycle regulatory protein, which is involved in cell cycle transition from the G0 to S phase. Interestingly that PPE markedly induced the expression of the cyclin D1 protein. In addition to the inhibitory effect of the signaling protein (Fig. 4B), all of them attenuated PPE-induced overexpression of cyclin D1. This indicates that the induction effect of PPE on osteoblast proliferation is likely associated with overexpression of cyclin D1 and is mediated by phosphorylation of ERK1/2, Akt, and JNK signaling.

DISCUSSION

Osteoblasts are mesenchymal cells that play a major role in osteogenesis, including synthesizing the bone matrix and coordinating the mineralization of the skeleton. This study demonstrates the effectiveness of PPE on osteogenic activity enhancement. PPE proficiently induced osteoblast proliferation and differentiation which was reflected by incrementally increased osteogenesis-related markers and mineralization.

Several bioactive compounds are components of PPE that have been extensively studied for their potential to improve skin care (Nagae *et al*., 2020), strengthen immune function (Lee *et al*., 2013), and play a role as an anti-antioxidant and improve metabolic functions (Nensat *et al*., 2021). Insulin-like growth factor-1 is a key regulator of fetal development found in the placenta, which is associated with the activation of type I collagen synthesis in osteosarcoma cells (Kudo *et al*., 1996). Active components in PPE that are entirely used in the present study are also characterized by LC-MS/MS and data analyzed against the domestic porcine *Sus scrofa* proteome database. The PPE in this study consists of 391 protein sequences from the entire 447 proteins. The researcher suggests the PPE in this study is mostly comprised signal-involved proteins, phosphoproteins, and disulphide-bond proteins (Padhomchai Pumbthongthae, 2020). This suggests that the signal-associated proteins may play an important function in the induction of osteoblast differentiation, such as insulin-like growth factor-1 and basic fibroblast growth factors.

With 60 μg/ml of PPE extract, osteoblast proliferation was induced without toxicity. The low concentrations of PPE exhibited bioactivities greater than higher concentrations, which may be associated with the toxicity from the high protein content of the extract. This is similar to other preparations of PPE in that 20–200 μg/ml of extract activates cell proliferation but results in higher concentrations of toxicity (Imamura *et al*., 2017). Moreover, the researchers also investigated the effect of PPE in various cell types including endothelial cells, fibroblasts, and keratinocytes in which PPE was found to exhibit bioactivity at low concentrations (data not shown). Therefore, 25 μg/ml of PPE was selected as the highest dose to investigate osteogenic activity on osteoblasts. As is well known, new bone formation is mainly mediated by osteoblasts which either increase the osteoblast proliferation or induce osteoblast differentiation (Ducy *et al*., 2000). PPE dramatically increased osteoblast proliferation on day 5 and slightly induced

it on day 7. The extract likely enhances osteoblast proliferation 5 days after maturation. As shown in Figure 3, PPE markedly enhanced ALP production and mineralization after 7 days. These phenotypes indicate the proficient property of PPE on osteoblast maturation and differentiation enhancement.

The induction of osteoblast differentiation was confirmed by the upregulation of osteogenic genes that are involved in bone formation markers. *Runx2* genes play an important function as transcription factors for other osteogenic proteins. Our findings demonstrate that PPE enhances the expression of *Runx2*, *ALP*, *COL1*, and *OCN* genes, indicating that PPE induced osteogenic maker through upregulation of *Runx2*. It has been shown that *Runx2* is the central regulator of bone formation, which is influenced by many signals such as fibroblast growth factor (Choi *et al*., 2005; Kim *et al*., 1998) and bone morphogenetic protein 2 (Lee *et al*., 2003). *Runx2*-deleted mice caused osteoblast deficiency and failed to form bone (Otto *et al*., 1997). Moreover, the level of functional *Runx2* is also responsible for bone loss in estrogen deficiency (Maruyama *et al*., 2007). Therefore, upregulation of *Runx2* by PPE enhancement could provide a therapeutic application in bone disorders.

Activation of growth factor receptors subsequently results in phosphorylation of the MAPK pathway. It has been reported that FGF induced ERK MAP kinase phosphorylation and later increased *Runx2* and was involved in the induction of osteoblast differentiation (Park *et al*., 2010). In addition to the PI3K-Akt signaling pathway, this signal has been implicated as a critical pathway for the differentiation of skeletal component cells, including chondrocytes and osteoblasts (Ghosh-Choudhury *et al*., 2002; Hidaka *et al*., 2001). A lack of Akt1 and Akt2 is the cause of delayed bone development (Peng *et al*., 2003). Interestingly, regulation of osteoblast differentiation and migration is cooperatively dependent on PI3K-Akt signaling and *Runx2*. The present study presented the effect of PPE on the induction of ERK and Akt phosphorylation. Moreover, PPE also upregulated the phosphorylation of JNK. This finding is in accordance with a previous study which showed that JNK is a key mediator of osteoblast differentiation and mineralization activity (Xu *et al*., 2017). Taking these data together, PPE regulated and enhanced the proliferation and differentiation of human osteoblasts mediated by either the ERK MAP kinase, PI3K-Akt, and JNK signaling pathways and/or subsequent upregulation of *Runx2* (Graphical Abstract). In this study, we intently present the osteogenic activities of PPE in terms of basic characteristics and main molecular mechanisms in human osteoblasts. Although an *in vitro* setting appropriately demonstrates the bioactivities of PPE, further information is still crucial for confirmation and development. The effects of the compound on other bone cells such as osteoclasts, bone matrix conformation, and *in vivo* models are urgently needed to prove the exhibition of the overall function of PPE in bone remodeling.

CONCLUSION

The present study demonstrated the bioactivity of PPE on human osteoblast activity, including cell proliferation, differentiation, and mineralization. In addition, the researchers confirmed the positive benefits of cell signaling investigation. These results suggest that PPE is a potential bioactive compound from agriculture industry waste that could enhance osteoblast activity for osteogenesis. Further investigation of active bioingredients of PPE and their effectiveness in animal models is required to improve clinical applications for bone disorders.

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AUTHORS' CONTRIBUTIONS

WS, CN, WK, and AJ originated and outlined experiments; WS conducted the experiments; WS, CN, and AJ interpreted the data; AJ, WK, TS, and RT provided laboratory materials. All authors contributed to manuscript preparation.

CONFLICTS OF INTEREST

The authors disclose that they have no conflicts of interest in this paper.

DATA AVAILABILITY

All the data generated in the study is included in the article itself.

ETHICAL APPROVAL

This study does not include any experiments on humans or animals.

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