치의과학박사 학위논문

Effects of Calcium Phosphates on Intracellular Multiple Signaling Pathways in Human Osteoblastic Cell Line

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치의과학과 치과생체재료과학 전공

정 윤 아

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Abstract

Effects of Calcium Phosphates on Intracellular Multiple Signaling Pathways in Human Osteoblastic Cell Line

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Objective

This study aims to identify the effect of calcium phosphates regulating multiple intracellular signaling pathways related to human osteoblast cell proliferation. Therefore, this study investigated the effect of three types of calcium phosphates on intracellular multiple signaling pathways involved in the proliferation of MG63 cells and investigated the effects of physicochemical changes of calcium phosphate due to sintering on intracellular calcium concentration and multiple intracellular signaling pathways.

Materials and methods

Hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and octacalcium phosphate (OCP) were used to examine the changes in the multiple intracellular signaling pathways. HA and β-TCP before and after sintering were used to study the effect of changes in the physicochemical properties of

calcium phosphate on cells due to sintering. As for the form of calcium phosphate, powdered calcium phosphate was used to maximize cell contact and ion release.

The physicochemical properties of calcium phosphates were analyzed using X-ray diffraction (XRD), Scanning Electron Microscope (SEM), and X-ray Photoelectron Spectroscopy (XPS).

Cell proliferation and biomineralization activity were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and alkaline phosphatase (ALP) assay. Intracellular multiple signaling pathways were confirmed by detecting individual signaling pathway proteins through western blotting. Intracellular Ca^{2+} level was measured in MG63 cells treated with or without calcium phosphate bonded to a fluorescent dye, Fura-2 acetoxymethyl ester (Fura-2 AM), using a high-speed wavelength converter and an inverted microscope.

Results

OCP promoted the p38 signaling pathway and suppressed the c-Jun N-terminal kinase (JNK) signaling pathway involved in the immune response. Src signaling pathway known to block the osteoblast differentiation factor runt-related transcription factor 2 (RUNX2) was inhibited by OCP. The protein kinase B (Akt) signaling pathway that plays an essential role in cell growth and metabolism was efficiently activated by OCP.

The HA, β-TCP, and OCP significantly increased osteoblast proliferation and biomineralization activity compared to the control group. However, intracellular multiple signaling pathways were different depending on the type of calcium phosphate. However, intracellular signaling pathways were activated differently depending on the types of calcium phosphates. As for intracellular Ca^{2+} levels, HA and β-TCP slightly decreased compared to the control, whereas OCP was measured at a level like that of the control.

Changes in the physicochemical properties of calcium phosphate by sintering significantly increased intracellular Ca^{2+} levels. In contrast, intracellular multiple signaling pathways showed mixed types of calcium

phosphate and sintering effects depending on the types of cell signaling pathways.

Conclusion

The three types of calcium phosphates with different physicochemical properties ultimately induce the proliferation of osteoblasts at similar levels but activate different intracellular signaling pathways depending on the type of calcium phosphate. In particular, the effect of sintering was observed only in specific intracellular signaling pathways, and the pattern differed depending on the type of calcium phosphate.

These results suggest that the physicochemical properties of calcium phosphate can act as factors regulating specific signaling pathways. In the future, in-depth research is needed to identify specific factors of calcium phosphate that affect intracellular signaling pathways by individually controlling various physicochemical properties of calcium phosphate.

The results of this study can be used as primary data for predicting changes in intracellular signaling pathways caused by physicochemical changes in calcium phosphate in the future. In addition, in various studies using calcium phosphates, it is thought that it can be used as primary data for research that promotes the bone regeneration effect by controlling specific intracellular signaling pathways along with drugs.

Keywords: Hydroxyapatite, β-tricalcium phosphate, Octacalcium phosphate, Intracellular signaling pathway, Intracellular calcium

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1. Introduction

1.1 Background

Bone, as a mineralized substance, is a vital component of maintaining and protecting the human body. Bone defects caused by injury, aging, inflammation, infection, and tumors significantly impact health and the ability to lead normal lives[1]. Every year, millions of bone grafting procedures are performed worldwide to treat severe bone abnormalities, placing a heavy financial burden on patient families and society as a whole[2].

It takes 6-12 weeks for bones to regenerate. During this period, complex physiological processes of bone formation and fracture healing occur. Numerous substances and circumstances influence bone regeneration[3], and the interaction of the matrix with the environment promotes a balance between osteoclasts and osteoblasts[4]. A critical bone defect more than 2 cm long or more than 50 % of its circumference cannot fully regenerate by self-growth and must be treated using biomaterials[5].

Biomaterials used in bone regeneration processes (bone graft materials), alone or in combination with other materials, promote bone healing responses by providing osteogenic, osteoconductive, or osteoinductive activity to localized site[6], and they also contribute to the restoration of morphological, physiological, and biomechanical functional roles.

Traditionally, autografts are the gold standard for bone graft materials[7]. They contain osteogenic bone cells, bone marrow cells, osteoinductive proteins, and osteoconductive collagen matrix that promotes the attachment and migration of new and existing bone cells[7]. However, autografts have the disadvantage of causing damage to healthy areas, such as pain and surgical complications (donor site morbidity) due to secondary surgery[8].

Alternatively, allografts from human cadavers or xenografts made from bovine or pig bones are other options; however, they have limitations due to supply and demand imbalance, high costs, immune response, and infection[9]. Various types of synthetic bone graft materials have been researched and

developed to compensate for the problems of existing bone graft materials. Synthetic bone materials, such as scaffolds, are applied in various ways and are increasingly used in bone regeneration. Although they exhibit poor osteogenic activity compared to autograft, synthetic bone graft materials with abundant sources offer diverse choices in structure, chemical/mechanical properties, and biological functions to meet the specific requirements of bone regeneration[10].

Calcium phosphates occupy an important position among the various biomaterials used in bone regeneration due to their similarity to the chemical components and substructures of natural bone tissue[11]. "CaP" refers to a large family of materials formed when Ca^{2+} and Pi react.

Calcium phosphates are biologically stable, biocompatible with the human body, and do not elicit immune response[8,9], making them widely used in clinical practice[12-14]. Calcium phosphates are categorized as degradable biomaterials, materials that can be absorbed into the biological environment. Calcium phosphates have been commercially used as scaffolds, bone cement, and bone graft.

The physicochemical properties of calcium phosphate, such as Ca/P ratio, solubility, surface roughness, specific surface area, crystallinity, surface charge, and surface energy, affect bone regeneration[15]. This literature review reviewed the effects of calcium phosphate on osteoblasts due to its physicochemical properties.

1.2 Review of literature

Many studies have found that calcium phosphates improve cell adhesion and proliferation, encouraging the formation of new bone minerals through interactions with extracellular matrix proteins[16]. Calcium phosphates can chemically dissolve under physiological conditions in the body and be reabsorbed by osteoclasts[17]. The space created by the dissolution of calcium phosphates under physiological conditions is replaced with new bone during

bone remodeling[18]. These bioactive properties enable calcium phosphates to be applied for various applications, including scaffolds, implants, coatings, and bone cement for bone regeneration[18].

The bioactivity of calcium phosphates exhibited different aspects depending on its species[19]. The variations in the Ca/P ratios of calcium phosphates affect how calcium and phosphate ions are released.

The viability of osteoblasts and osteoclasts, the formation of bone minerals, and the expression of genes related to osteogenic differentiation of osteocytes (such as collagen type 1, osteopontin, osteocalcin (OCN), alkaline phosphatase (ALP), bone sialoprotein (BSP), runt-related transcription factor 2 (RUNX2), and bone morphogenetic proteins) are all enhanced by the pH change of the local microenvironment of the bone caused by the released calcium and phosphate ions[14,20]. Ca^{2+} and Pi released from calcium phosphate play various roles in bone formation in living organisms.

Ca^{2+} is a significant ion constituting the bone matrix, a factor that affects cells and biological systems in various ways[21]. Ca^{2+} plays a role in the formation and maturation of bone tissue through calcification and influences bone cell maturation and bone regeneration by regulating cell signaling pathways[22,23]. Various studies have reported that Ca^{2+} stimulates the bone synthesis pathway of osteoblasts through activation of ERK1/2[24] and extends the lifespan of osteoblasts by activating the PI3K/Akt signaling axis[25,26].

Ca^{2+} is involved in bone regeneration, a versatile second messenger influencing many cellular signaling processes[27]. Various intracellular signaling pathways are activated by intracellular Ca^{2+}, such as the extracellular signal-regulated kinases (ERKs)[28] and calmodulin-dependent kinases (CaMKs)[29].

Pi are the major constituents of the bone matrix, and the absence or abnormal absorption or reabsorption of Pi leads to mineral loss and the formation of the unmineralized bone characteristic of osteomalacia or rickets[30]. Pi significantly regulates chondrocyte differentiation, bone resorption, osteoblast proliferation, and bone mineralization[31]. Pi treatment of human osteosarcoma U2OS cells led to a suppression of cell growth and a decrease in

intracellular cAMP levels and adenyl acid cyclase activity[32].

High extracellular Pi concentration inhibits both osteoclast differentiation and bone resorption activity. Additionally, it has been demonstrated that extracellular Pi decreases osteoclast activity, at least in part, by directly inducing osteoclast death. Pi inhibits osteoclast activity by promoting osteoprotegerin production in osteoblastic cells[15,33]. In many cells, including but not limited to osteogenic cells, Pi alters gene expression or cellular function[34]. As a sample, it is reported to impact the control of RUNX2 and OCN in human vascular smooth muscle cells[35]. Furthermore, Previous studies have demonstrated that Pi controls organ growth via Akt/mTOR pathway and rapidly accelerated fibrosarcoma (Raf) / mitogen-activated protein kinase (MEK) /extracellular-signal-regulated kinase (ERK) pathway[36].

Calcium phosphate's physical and chemical properties significantly impact cells adhering to one another and forming tissues by changing the surface adsorption of extracellular matrix protein. As a result, it affects bone mineral formation[37]. Specifically, surface properties of calcium phosphate, such as surface roughness, crystallinity, solubility, phase content, porosity, and surface energy, strongly influence cell adhesion and the ability to absorb extracellular matrix proteins[38].

The degradation of calcium phosphates and ion release increases the local concentration of Ca^{2+} and Pi, promotes bone mineral synthesis on the calcium phosphates surface, and contributes to osteoblast adhesion, proliferation, and new bone formation[19].

The solubility of the calcium phosphate phase at 37 °C −log (Ks) is β-tricalcium phosphate (β-TCP) (29.5) >> octacalcium phosphate (OCP) (95.9) >> hydroxyapatite (HA) (117.2), and relative solubility in acidic buffer decreases in the order of amorphous calcium phosphate (ACP) >> α-tricalcium phosphate (α-TCP) >> β-TCP >> calcium-deficient hydroxyapatite (CDHA) >> HA[19,39]. The low-soluble calcium phosphates have low ion exchange with the surroundings and a slow recrystallization rate at the surface. In contrast, high-soluble calcium phosphates induce rapid changes in local pH and ion concentration and promote the formation of low-crystalline carbonate

apatite[40]. In a previous in vitro study, the Ca^{2+} level of the culture medium incubated with calcium phosphate for 72 h was 1.3 to 1.5 mM for HA, β-TCP, and dicalcium phosphate dihydrate (DCPD), whereas OCP was relatively low at 0.5 to 0.6 mM. The concentration of Pi gradually increased to 16.2 mM after 72 h of culturing only DCPD, and the other calcium phosphates maintained a low concentration of about 0.3 mM. The low Ca^{2+} concentration of OCP inhibited the initial adhesion of osteoblast ST-2 cells, and the increased Pi concentration of DCPD over time led to apoptosis of ST-2 cells. HA and β-TCP showed superior initial cell proliferation compared to OCP. Still, after about 21 days, cell proliferation of the three types of calcium phosphates, except for DCPD, was at a similar level[41]. In a previous in vivo study, there was no difference in promoting new bone formation between calcium phosphates with high and low solubility seven days after bone graft. Furthermore, in the early stage of transplantation, the promotion of new bone formation was slow at low Ca^{2+} concentrations. However, at about 21 days, new bone was formed at the same concentration as calcium phosphate, maintaining high Ca^{2+} concentration even when low Ca^{2+} concentration was maintained[43].

The crystallinity and solubility of calcium phosphates not only affect the level of adsorbed proteins but can also affect cell adhesion by influencing the pH and level of ions in the medium. Calcium phosphates (Nano HA) with high crystallinity showed a significantly higher proliferation promoting the bone marrow mesenchymal stem cells than ACPs (Nano ACP)[43]. In the solubility test, α-TCP pellets showed better rat Bone marrow-derived stem/stromal cell (BMSCs) attachment and proliferation than rapid resorbable calcium phosphate ceramic consisting of $CaKPO_4$ disc-shaped pellets (sample code R5) because calcium ions are depleted as large amounts of phosphate and potassium ions are simultaneously released from $CaKPO_4$ pellets[44].

The porosity of calcium phosphates affects osteoblast proliferation by protein adsorption. The increased porosity of calcium phosphates improves the surface area in contact with the fluid, speeding up the dissolution rate[45]. A pore size of 20-500 μm enhanced the protein adsorption of calcium

phosphates[46].

The surface area of calcium phosphates affects osteoinductivity[47]. Previously studied biphasic calcium phosphate A (BCPA) and biphasic calcium phosphate B (BCPB) ceramics were compared. Two materials had the same chemical composition and macrostructure, but the surface area of BCPA was five times higher[48]. BCPA with a high specific surface area induced bone formation in all animals, whereas BCPB did not induce bone formation. It also depends on the type of material with an optimal specific surface area that induces maximum osteoinductiviy[48].

The surface roughness of calcium phosphates is a factor that affects the adhesion, proliferation, and differentiation of osteoblasts. In previous studies, it has been reported that increasing calcium phosphate surface roughness increases the proliferation of osteoblasts[49]. Surface roughness is determined by the grain size and particle size of calcium phosphates, and roughness affects osteogenesis-related protein[16] and cell adhesion on the surface of calcium phosphates[49]

The surface energy of calcium phosphate affects osteoblast proliferation by protein adsorption[50] and osteoblast adhesion[51]. In previous studies, the surface energy reduction of HA and β-TCP polar components decreased albumin and fibronectin adsorption and increased cell adhesion. Nanotopography strongly affects the protein adsorption process more than the calcium phosphate chemistry[49].

The surface charge affects osteoblast proliferation. In previous studies, the polarization method changed the surface charge to a negatively charged surface because it was assumed that a negatively charged surface promotes cytokine or growth factor adhesion[52]. Among the calcium phosphates, HA, β-TCP, and OCP are currently the most used clinical materials.

HA is the most prevalent biomineral crystalline phase, accounting for ~70% of human bone, and has been extensively used for bone regeneration[53]. The chemical formula of HA is $Ca_{10}(PO_4)_6(OH)_2$, and the Ca/P ratio is 1.67. HA adopts a hexagonal phase in a physiological environment, which is a stable structure. Therefore, it is the most stable among the calcium phosphate

family and has low solubility[54]. The surface of HA provides nucleation sites for the precipitation of apatite crystals (in culture medium, saturated with calcium ions and phosphate ions)[55,61].

HA has long-term in vivo stability and osteoconductive properties, and the physicochemical and biological properties can be controlled by replacing the Ca/P ratio and F^-, Cl^-, CO_3^{2-}, Mg^{2+}, $Sr^{2+,}$ and other ions[55]. The mechanical properties of HA reach their maximum at a Ca/P ratio of 1.67, above which the strength decreases[56]. Various studies have shown that the crystallization of HA was inhibited by substitution with Mg^{2+} ions, resulting in the formation of fewer large crystals and a more significant number of apatite nuclei, resulting in improved biological activity[56]. Substitution with F^- ions enhances the stability of HA[57].

Chemically, sintered HA is categorized as being reasonably stable and has been demonstrated to not disintegrate in bone defects over an extended length of time; nonetheless, it offers higher biocompatibility with the regeneration tissue[58].

The chemical formula of β-TCP is β-$Ca_3(PO_4)_2$, and β-TCP has a Ca/P ratio of 1.5. β-TCP is a high-temperature phase typically obtained by the thermal conversion of ACP[59]. β-TCP has a crystal structure of a rhombohedral space group[59]. β-TCP is less soluble than OCP[60] or DCPD[61], which are generally considered at equilibrium in the physiological fluids of the human body. In vitro results demonstrated that β-TCP is insoluble in simulated body fluid (SBF) at pH 7.4[62], and possibly at pH 6.0[63]. β-TCP is resorbed by macrophages and multinucleated giant cells by a cell-mediated process and does not spontaneously dissolve in vivo[62].

β-TCP enhanced the proliferation of osteoblasts due to its nanoporous structure[64]. In previous studies, the application of β-TCP significantly increased the bone regeneration in bone defects of animal models compared to nanostructured carbon implants and porous titanium[65]. It demonstrated a significantly higher bone regeneration compared to bone autografts[66].

β-TCP has higher osteoconductivity and osteoinductivity than HA on Bone marrow-derived stem/stromal cells (BMSCs)[67]. In a previous study,

porous β-TCP can enhance the formation of new bone by increasing the dissolution and absorption of substances and promoting the infiltration of MSCs[68]. β-TCP with macro and microparticles of < 7 μm removed using argon glow discharge plasma (GDP) promoted human mesenchymal stem cell (hMSC) proliferation and osteoblast differentiation and was more effective than untreated β-TCP to increase the formation of new bones[69].

The chemical formula of OCP is $Ca_8 H_2(PO_4)_6 5H_2O$, and OCP has a Theoretical Ca/P molar ratio of 1.33. Depending on the synthesis conditions, OCPs may not be stoichiometric, and their structure can be calcium excess (Ca/P = 1.48) or a calcium deficiency (Ca/P = 1.23)[70].

The structure of OCP is alternately layered with hydration layers[97]. Based on this structure, it has been proposed as a precursor of bioapatite crystals such as bones and teeth[71]. Although a very similar structure exists between OCP and HA, OCP is more unstable than HA and is known to be hydrolyzed to HA, but the mechanism still needs to be elucidated[71]. The hypotheses proposed as the hydrolysis mechanism of OCP are the dissolution-reprecipitation mechanism[72] and the ionic diffusion-crystallization transition hypothesis[73].

The hydrolysis rate of OCP is significantly different between in vivo and vitro[70]. While the structural transfer of OCP in vitro was complete within a few hours[74], OCP transplanted into mouse calvarial defects hydrolyzed very slowly after 21 days[75]. OCP stimulates bone mineralization by stimulating osteoblasts and osteoclasts and producing an early bone matrix structure that mimics the formation of morphological bone[76]. The early bone matrix structure of OCP differs from that of other calcium phosphates. Cuboidal osteoblasts aligned around the implanted OCP granules initiate a non-calcified osteoid bone matrix composed of OCP particles and non-collagenous proteins[77]. The structure of this initial bone matrix is composed of fine filaments and granular materials within a non-collagen matrix[77], which is almost identical to that of bone nodules[78]. Early bone deposition of OCPs mimicking morphological bone formation is one of the properties of OCPs not observed with other calcium phosphates[79].

Another distinctive characteristic of OCP is how cells react with OCP to induce the production of osteoclasts by stimulating osteoblasts in vitro[80]. Compared to HA, OCP dramatically increased the differentiation of ST-2 cells into osteoblasts[81]. ALP and osterix are osteoblast differentiation indicators upregulated due to OCP determination. OCP-mediated differentiation and mRNA expression of ALP, osterix, and type I collagen were also dose-dependently improved[81]. When osteoblasts and osteoclasts were cultured with OCP in a culture medium without $1,25(OH)_2D_3$, an essential factor for upregulating osteoclasts, osteoblast activation was achieved by expression of osteoblast NF-κB ligand (RANKL), resulting in osteoclast formation[82]. It was observed that the induced osteoclasts were relatively firmly attached to the OCP surface[82]. The activity of tartrate-resistant acid phosphatase (TRPA, histochemical marker for osteoclasts)-positive osteoclasts was observed around OCP granules. It was confirmed that OCP is biodegraded by a cell-mediated resorption process initiated by osteoclasts and promotes bone formation by inducing the activation of osteoclasts and osteoblasts[76].

Accordingly, it can be seen that OCP acts as an up-regulator of osteoblasts, induces osteoclasts, and is biodegradable by physicochemical alterations such as Ca^{2+} consumption and inorganic phosphate ion release, as well as increased adsorption affinity of serum proteins such as α-2-HS-glycoprotein, apolipoprotein (proteins involved in bone metabolism)[83].

1.3 Objective

Cells detect physical signals, such as electricity, temperature, and light, and chemical signals, such as small molecules that bind to cell surfaces. The detected signals induce cell responses such as differentiation, proliferation, and death through intracellular signal transduction pathways depending on external stimuli.

For this reason, the type and characteristics of calcium phosphate change protein adsorption, cytokine secretion, cell adhesion, etc., which induces changes in intracellular signaling pathways, leading to responses such as

proliferation, differentiation, and death of cells. Studies so far have mainly focused on consequential research methods that observe cell adhesion and protein adsorption through changes in the properties of calcium phosphate or observe only cell proliferation and differentiation.

However, even if a material with excellent cell adhesion and protein adsorption was developed, there was a problem that it could not be used because cell proliferation and differentiation did not occur. A mechanism for changes in intracellular signaling pathways caused by calcium phosphate is needed to solve these problems.

Thus, this study investigated the effect of calcium phosphate on intracellular multiple signaling pathways involved in the proliferation of MG63 cells and investigated the effects of physicochemical changes of calcium phosphate due to sintering on intracellular calcium concentration and multiple intracellular signaling pathways. The calcium phosphate used in the evaluation was powder to maximize cell contact and ion release.

2. Materials and Methods

2.1 Influence of OCP on various cell signaling pathways

2.1.1 Preparation of OCP

OCP powder was prepared using a heterogeneous synthesis method. 5 g of DCPD (Junsei, Tokyo, Japan) was mixed with 1,000 mL of distilled water as the starting material, and then the mixture was steadily heated with a magnetic stirrer at 200 rpm until it reached 80 °C. The pH was then adjusted to 5. OCP began to precipitate, and the reaction was completed in about ten minutes. The precipitate was filtered through Whatman filter paper 2, washed with ethanol, and dried in an oven at 80 °C for 24 h.

2.1.2 Characterization of OCP

SEM analysis was performed to observe the microstructure of the sample. The surface morphology of OCP was analyzed with a field emission scanning electron microscope (FE-SEM (S-4800 Hitachi, Tokyo, Japan). The samples were observed at x1,000 and x10,000 magnifications under 15 kV. The specimens were prepared by pressing and pulverizing powder dust to prevent damage to the detector due to vacuum during SEM observation.

XRD analysis was performed to identify the sample through the diffraction pattern analysis of OCP. The XRD analysis was performed using XRD (Aeris, Malvern, UK) with a Cu-K radiation source at a scan rate of 2.0/min and a step size of 0.022°.

XPS analysis was performed to confirm the ratio of Ca/P through component analysis of OCP. The chemical elements in OCP were analyzed using a Kα XPS spectrometer (K-Alpha, Thermos Scientific, Waltham, USA). Before XPS analysis, the sample surface was sputter-cleaned using argon ions to eliminate impurities. The peaks of the C1s, O1s, Ca2p, and P2p were used to calculate the concentrations. Additionally, the quantities of different surface elements were measured.

2.1.3 Cell preparation

MG63 cells were used to confirm the effect of the signaling pathway in osteoblasts by OCP. MG63 human osteoblast cell line was purchased from Korea Cell Line Bank (Seoul, South Korea).

The cells were maintained in Dulbecco's Modified Eagle medium (DMEM, Gibco, Waltham, USA) supplemented with 1% penicillin-streptomycin (Gibco, Waltham, USA) and 10% fetal bovine serum (FBS, Gibco, Waltham, USA) at 37 °C and 5 % CO_2.

OCP powder was added at 10 or 20 mg/mL to each well of a 6-well cell culture plate (SPL Life Sciences, Pocheon, Korea) and incubated for 24 - 48 h at 37 °C and 5 % CO_2. Subsequently, MG63 cells were seeded into the 6-well cell culture plate with DMEM, containing 10 % FBS and 1 % penicillin-streptomycin and incubated for 72 h at 37 °C and 5 % CO_2

2.1.4 Inhibitors

Inhibitors are used to determine whether the effects of calcium phosphate are suppressed. The inhibitors were used as follows. SB203580 (30 μM, Sigma Aldrich, St. Louis, USA), and PD98509 (25 μM, Selleckchem, Houston, USA).

2.1.5 MTT assay

The cell proliferation was studied by MTT assay after 5, 7, 11, and 13 days of cell seeding on OCP and control (MG63 cell without OCP). MG63 cells treated with OCP and control were seeded in 96-well plates at a density of 3×10^5 in an incubator maintained at 37 ºC and 5 % CO_2.

20 μL of MTT solution (10 %, biosesang, Seongnam, South Korea) diluted in phosphate-buffered saline (PBS) was added to each well. The 96-well plates were wrapped in aluminum foil to block light and incubated at 37 ºC, 5% CO_2 for 4 h. After removing the supernatant, 20 μL of dimethyl sulfoxide was added to each well and left at room temperature for 15 min.

The absorbance of each well was then measured at 595 nm using an enzyme-linked immunosorbent assay plate reader (Sunrise, Tecan, Morgan

Hill, USA). All measurements were performed thrice to ensure test repeatability.

2.1.6 ALP assay

Biomineralization by OCP was assessed using the ALP assay after 5, 7, 11, and 13 days of cell seeding on OCP and control. ALP activity was determined by the manufacturer's instructions using an ALP assay kit (Colorimetric, catalog number ab83369, Abcam, Waltham, USA). MG63 cells treated with or without OCP were seeded in 96-well plates at a density of 3×10^5 in an incubator maintained at 37 ºC and 5 % CO_2.

MG63 cells were homogenized in assay buffer after being washed with cold PBS. For each sample, supernatants were put into 96-well plates (SPL Life Sciences, Pocheon, Korea), and assay buffer was used to fill each well to a total volume of 80 μL. P-nitrophenyl phosphate solution was added to the sample wells, and ALP enzyme solution was added to the standard wells only. The 96-well plates were covered with aluminum foil to block light and incubated for 60 min. After incubation, 20 μL of the stop solution was added.

A standard curve was created according to the manufacturer's instructions. By comparing the standard curve to the sample curve, the ALP activity of each sample was calculated. Finally, the absorbance was evaluated at 405 nm using a SpectraMax ABS PLUS Absorbance Microplate Reader (Molecular Devices, San Jose, USA). All measurements were performed thrice to ensure test repeatability.

2.1.7 Western blotting

Western blotting was performed to confirm intracellular signaling pathways by OCP. The cells cultured by the method described in Section 2.1.3 were harvested and centrifuged at 15,000 g for 30 min at 4 °C.

The protein concentration was quantified by separating the supernatant from the cells. Proteins were eluted using a 4x concentrated lithium dodecyl sulfate sample buffer that had been treated with β-mercaptoethanol at 70 °C for 15 min.

Proteins were separated using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the mini-Protean Tetra Handcast System (Bio-Rad Laboratories, Hercules, USA) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5 % non-fat dry milk in PBS-Tween20 (PBS-T) and incubated with primary antibodies at 4 °C overnight.

After washing five times with PBS-T, the membranes were incubated with anti-rabbit Immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody (1:4,000) or anti-mouse IgG, HRP-linked antibody (1:4,000). Protein bands were detected using the SuperSignal West Femto Maximum Sensitivity Substrate (34095, Thermo Fisher Scientific, Waltham, MA, USA).

The primary antibodies were as follows: anti-p38 (1:1,000, 9212S), anti-phospho-p38 (1:1,000, 4511S), anti-SAPK/JNK (1:1,000, 9252S), anti-phospho-SAPK/JNK (1:1,000, 9251S), anti-Akt (1:1,000, 9272S), anti-phospho-Akt (Ser473) (1:1,000, 9271S) and anti-β-actin (1:1,000, 3700S). All antibodies were purchased from Cell Signaling Technology (Danvers, USA). Anti-Raptor/mTORC1 antibody (1:1,000, sc-81537) was purchased from Santa Cruz Biotechnology (Dallas, USA).

2.1.8 Statistical analysis

Image J was used in the studies of protein level analysis (US National Institutes of Health). Student's t-tests were performed for the statistical analysis. $p < 0.05$ was defined as statistically significant, and all values were expressed as the mean ± standard error of the mean (SEM).

2.2 Regulation of intracellular multiple pathways by three calcium phosphates

2.2.1 Preparation of calcium phosphates

The non-sintered HA samples were prepared by hydration utilizing OCP as a

starting material. The non-sintered β-TCP powder was prepared from reagent-grade TCP powder (Sigma Aldrich, St. Louis, USA). OCP powder was prepared the same way as described in Section 2.1.1

2.2.2 Cell preparation

MG63 cells were used to confirm the effect of the signaling pathway in osteoblasts by three types of calcium phosphates. Cell preparation was performed in the same way as in Section 2.1.3. However, 4 or 10 mg/mL of calcium phosphates were treated.

2.2.3 Inhibitors

The inhibitor determines whether calcium phosphates inhibit the intracellular signaling pathways. The inhibitors were used as follows. SB203580 (30 μM, Sigma Aldrich, St. Louis, USA), SP600125 (10 μM, Selleckchem, Houston, USA), PD98509 (25 μM, Selleckchem, Houston, USA), and wortmannin (20 μM, Sigma Aldrich, St. Louis, USA)

2.2.4 MTT assay

Cell proliferation was studied by MTT assay after 5, 7, 11, 12, and 13 days of cell seeding on calcium phosphates and control (MG63 cell without calcium phosphates). The evaluation method was the same as in Section 2.1.5.

2.2.5 ALP assay

ALP activity was evaluated by ALP assay after 5, 7, 11, 12, and 13 days of cell seeding on calcium phosphates and control (MG63 cell without calcium phosphates). The evaluation method was the same as in Section 2.1.6.

2.2.6 Intracellular Ca^{2+} analysis

Intracellular Ca^{2+} level was measured to confirm the effect of three types of calcium phosphates. A 12 mm coverslip was coated with poly-L-lysine for 24 h and then washed three times with PBS. MG63 cells treated with calcium

phosphates and controls were incubated in 24-well plates with 12 mm coverslips.

An intracellular Ca^{2+} indicator, 5 μM of Fura-2 AM, and 0.01% pluronic F-127 in Normal Tyrode solution were added to each well and incubated at 37 °C for 30 min. A solution for measuring intracellular Ca^{2+} was prepared by adding 140 mM NaCl, 5 mM KCl, 10 mM Hydroxyethyl piperazine Ethane Sulfonic acid (HEPES), 10 mM glucose, and 1 mM $MgCl_2$. Fura-2 AM loaded cells were mounted in a perfusion chamber on the inverted microscope (Eclipse Ti, Nikon, Japan).

The Fura-2 AM load cell is excited with a Lambda DG 4 ultra-high-speed wavelength switching light source (Sutter Instrument, Novato, USA) at 340 and 380 nm in fast switching mode and emission. After collecting the fluorescence signal at 510 nm and setting the region of interest (ROI) and background, the fluorescence intensity was measured using the time measurement function.

Data were analyzed using image analysis software (MetaFlour, Molecular device, San Jose, USA) for each region of Fura-2 AM loaded cells.

2.2.7 Western blotting

Western blotting was performed to confirm the intracellular signaling pathways of three types of calcium phosphates. Western blotting experiments were performed in the same as described in Section 2.1.7.

The primary antibodies were as follows: anti-p38 (1:1,000, 9212S), anti-phospho-p38(1:1,000, 4511S), anti-SAPK/JNK (1:1,000, 9252S), anti-phospho-SAPK/JNK (1:1,000, 9251S), anti-Akt (1:1,000, 9272S), anti-phospho-Akt (Ser473) (1:1,000, 9271S), anti-EGFR (1:1000, 2232S), anti-SAPK/JNK (1:1,000, 9252S), anti-phospho-SAPK/JNK (1:1,000, 9251S), anti-phospho-EGFR (1:1000, 2236S), and anti-Raptor/mTORC1 (1:1,000, 2280S). All antibodies were purchased from Cell Signaling Technology (Danvers, USA).

2.2.8 Statistical analysis

Image J was used in the studies of protein level analysis (US National Institutes of Health). Unpaired two-tailed t-test were performed for the statistical analysis.

$p < 0.05$ is defined as statistically significant, and all values were expressed as the mean ± SEM.

2.3 Effects of sintering on intracellular multiple signaling pathways

2.3.1 Preparation of calcium phosphates

The non-sintered HA and the β-TCP powder are the same as described in Section 2.2.1. The sintered HA powder was made by heating reagent-grade HA powder (Junsei, Tokyo, Japan) at 1200 °C for 2 h, followed by crushing. Moreover, commercially available β-TCP powder (Himed, NY, USA) was used as the sintered β-TCP powder.

2.3.2 Characterization of calcium phosphates

SEM analysis was performed to observe the microstructure of the sample. The surface morphology of HA and β-TCP before and after sintering were analyzed with an FE-SEM (S-4800 Hitachi, Tokyo, Japan). The samples were observed at x1,000 and x5,000 magnifications under 15 kV. The specimens were prepared by pressing and pulverizing powder dust to prevent damage to the detector due to vacuum during SEM observation. XRD and XPS analysis were evaluated in the same way described in Section 2.1.2.

2.3.3 Cell preparation

MG63 cells were treated with 10 mg/mL of calcium phosphate to confirm the changes in intracellular multiple signaling pathways in osteoblasts by HA and β-TCP before and after sintering. Cell preparation was performed in the same

as described in Section 2.1.3.

2.3.4 Inhibitors

The inhibitors used are the same as in Section 2.2.3.

2.3.5 Western blotting

Western blotting was performed to confirm the changes in intracellular multiple signaling pathways in osteoblasts by HA and β-TCP before and after sintering. Western blotting experiments were performed in the same way described in Section 2.1.7.

2.3.6 Intracellular Ca^{2+} analysis

The effect of changes in physicochemical properties due to sintering on intracellular Ca^{2+} was confirmed using before and after sintering HA and β-TCP.

Intracellular Ca^{2+} analysis was performed in the same as described in Section 2.2.6.

2.3.7 Statistical analysis

Image J was used in the studies of protein level analysis (US National Institutes of Health). One-way ANOVA was performed for the statistical analysis. $p < 0.05$ is defined as a statistically significant difference, and all values were expressed as the mean ± SEM.

3. Results

3.1 Influence of OCP on various cell signaling pathways

3.1.1 Characteristics of OCP

From the SEM results, it is observed that characteristic OCP takes the form of ribbons with size variations of several tens of micrometers. Specific peaks identifying OCP could be seen in the XRD pattern images. Inorganic crystal structure database (ICSD) data (#04-016-3473) was used to analyze the XRD pattern. The Ca/P molar ratio analyzed by XPS was 1.23. The SEM, XRD, and XPS patterns of the synthetic OCP are displayed in Fig. 1.

3.1.2 OCP stimulated cell proliferation and biomineralization

An MTT assay was used to examine whether OCP enhances cell proliferation. The MTT assay findings at 5, 7, 11, and 13 days showed that OCP-treated cells (10 or 20 mg/mL) had increased cell proliferation. There was a statistically significant difference between the experimental group and the control group ($p < 0.05$) (Fig. 2A).

ALP assay was performed to confirm that OCP promotes biomineralization. Experimental conditions were the same as for MTT. The ALP activity of MG63 cells cultured with OCP significantly increased ($p < 0.05$) (Fig. 2B). These results suggest that OCP can increase the activity of osteoblasts by upregulating factors that increase cell proliferation and biomineralization activity in MG63 cells.

3.1.3 OCP facilitated the p38 signaling pathway

The expression of the p38 protein was evaluated in MG63 cells to ascertain whether OCP impacted the p38 signaling pathway. Total p38 protein levels in cells with and without OCP treatment were identical.

However, phospho-p38 protein expression was statistically higher in the 10 or 20 mg/mL OCP treated group than in the control group ($p < 0.05$). The

amount of detected phospho-p38 protein expression was proportional to the amount of OCP (Fig. 3A).

3.1.4 OCP inhibited the Src signaling pathway

The expression of the Src protein was evaluated in MG63 cells to ascertain whether OCP impacted the Src signaling pathway. The total amount of Src protein expression was the same with or without OCP treatment.

However, MG63 cells treated with 10 or 20 mg/mL OCP had significantly decreased phospho-Src protein expression than the control group ($p < 0.05$). The amount of detected phospho-Src protein was inversely proportional to the amount of OCP (Fig. 3B).

3.1.5 OCP attenuated the JNK signaling pathway

The total JNK protein levels were slightly decreased upon OCP treatment. Phospho-JNK protein expression was statistically decreased than the control ($p < 0.05$). The amount of phospho-JNK protein expression detected in OCP 10 or 20 mg/mL was similar. JNK inhibitor PD98509 (25 μM) completely inhibited OCP induced phospho-JNK (Fig. 4).

3.1.6 OCP promoted the Akt signaling pathway

OCP treatment affected the changes in the Akt signaling pathway of MG63 cells. Total Akt protein expression was significantly reduced in the OCP-treated group compared to the control group ($p < 0.05$).

However, phospho-Akt Ser 473 protein expression was increased in OCP treated group than in the control group. The total amount of Akt protein expression was proportional to the amount of OCP, and the amount of phospho-Akt S473 protein expression was inversely proportional to the amount of OCP (Fig. 5).

3.2 Regulation of intracellular multiple pathways by three calcium phosphates

3.2.1 Results on cell proliferation

The proliferation of MG63 cells treated with calcium phosphates was significantly higher than the control group on every observation day ($p < 0.05$). Cell proliferation increased to a maximum on 11 days of culture. Cell proliferation according to types of calcium phosphates were 266.4 %, 304.4 %, and 275.6 % for HA, β-TCP, and OCP, respectively, with β-TCP showing the highest proliferation. However, the difference between groups was not statistically significant (Fig. 6A).

3.2.2 Results on biomineralization

The ALP activity of three types of calcium phosphates treated groups was five times higher than that of the control group after 5 days. There was a significant difference between the experimental groups and the control ($p < 0.05$). The ALP activity was almost no different according to the three types of calcium phosphates (Fig. 6B).

3.2.3 Results on intracellular Ca^{2+} levels

The intracellular calcium level plays a vital role in the intracellular signaling pathway. The intracellular Ca^{2+} level of MG63 cells exposed to HA and β-TCP was decreased compared to the control group ($p < 0.05$). However, the group exposed to OCP showed similar intracellular Ca^{2+} levels to the control group. There was no significant difference in intracellular Ca^{2+} levels of HA and β-TCP (Fig. 7).

3.2.4 Results on the p38 signaling pathway

The results showed no distinction in total p38 protein levels between the three types of calcium phosphates treatment groups and the control group. However, depending on the kind of calcium phosphates, there was a significant change

in the protein of phospho-p38 ($p < 0.05$). In particular, 4 mg/mL of β-TCP and OCP enhanced the expression of phospho-p38 more than three times than HA. The phospho-p38 protein expression of low-dose HA was similar to that of the control group. The expression of the phospho-p38 protein was decreased in cells treated with the inhibitor (Fig. 8).

3.2.5 Results on the JNK signaling pathway

JNK protein was expressed in all calcium phosphates treated groups. However, there was barely any expression of phospho-JNK, an active version of JNK. OCP and β-TCP treatment groups (4 and 10 mg/mL) showed negligible protein expression levels ($p < 0.05$). In contrast, HA showed little protein expression. JNK activity was inhibited when SP600125 was used (Fig. 9).

3.2.6 Results on the Akt signaling pathway

Total Akt protein expression caused by the three types of calcium phosphate showed a statistically significant decrease ($p < 0.05$). Total Akt protein expression level was highest in the control group, low in the β-TCP and OCP-treated groups, and weak in the HA-treated group.

However, in the activated Akt state, phospho-Akt S473, the highest protein expression was observed in the OCP-treated group. The increase in phospho-Akt S473 protein expression in the OCP-treated group was statistically significant ($p < 0.05$). In the β-TCP-treated group, the protein expression level was very low, and the expression level was similar to that of the control group. Protein expression was hardly seen in the HA-treated group. Still, the level was significantly lower than that of the control group (Fig. 10).

3.2.7 Results on the EGFR signaling pathway

Total EGFR protein did not change after treatment with calcium phosphates. However, the level of phospho-EGFR, an active version of EGFR, was significantly reduced when treated with calcium phosphates ($p < 0.05$). Although the HA group showed slightly increased phospho-EGFR protein expression compared to the β-TCP and OCP groups, there was no statistically

significant difference (Fig. 11A).

3.2.8 Results on the Raptor/mTORC1 signaling pathway

The impact of calcium phosphates was studied about changes in the Raptor/mTORC1 signaling pathway. Protein expression was observed in the control and experimental groups treated with HA, β-TCP, and OCP. Still, there was no significant difference between the groups (Fig. 11B). However, this result is for total Raptor protein, and further studies on the protein expression of phospho-Raptor in an activated state are needed.

3.3 Effects of sintering on intracellular multiple signaling pathways

3.3.1 Characteristics of the HA and β-TCP before and after sintering

The XRD patterns of the sintered and non-sintered HA powders showed a HA phase, although the non-sintered powder had a low level of crystallinity (Fig. 12A, 12B). The variations in their crystalline characteristics may have impacted their bioactivity or biodegradation. The existence of β-TCP was also identified through XRD pattern analysis. As a result, the crystallinity of the sintered β-TCP powder and the non-sintered β-TCP powder was analyzed to be similar (Fig. 12C, 12D). These results are expected to show similar biological activity and biodegradation rates. The XRD patterns result is consistent with the ICSD data (HA #00-009-0432, β-TCP #00-009-0169).

The sintered HA powder prepared at high temperatures forms a clear particle of HA. In contrast, the non-sintered HA powder prepared at a low temperature had a low crystallinity and an unclear particle form. The two β-TCP powders possessed similar distinct HA particles due to similar crystallinity. From SEM analysis, it was observed that the non-sintered sample was in the form of agglomerated particles without necking and that the

sintered sample had necking due to surface energy reduction due to thermal energy. To investigate the Ca/P molar ratio, FE-SEM energy dispersive spectroscopy measurements were used. The ratio obtained for non-sintered HA was 1.38, whereas, for the sintered, it was 1.72. Similarly, for non-sintered and sintered β-TCP, it was 1.39 and 1.48, respectively. Additionally, we obtained the FE-SEM images at x1,000 and x5,000 magnifications for calcium phosphates identification. To investigate the constituents of each material, the energy of the emitted photoelectrons was measured using XPS.

The Ca/P ratio obtained from the XPS spectrum for non-sintered HA was 1.29, whereas, for the sintered, it was 1.55. Similarly, for non-sintered and sintered β-TCP, it was 1.23 and 1.24, respectively (Fig. 13A).

3.3.2 Effect of sintering on intracellular Ca^{2+} levels

The non-sintered HA lowered the intracellular Ca^{2+} level significantly compared to the control group ($p < 0.05$). However, the sintered HA showed no change in the intracellular Ca^{2+} level. β-TCP showed the same pattern as HA. However, non-sintered β-TCP had a statistically significant lower intracellular Ca^{2+} level than the control group ($p < 0.05$) (Fig. 13B).

3.3.3 Effect of sintering on the JNK signaling pathway

HA and β-TCP significantly inhibited the JNK signaling pathway compared to the control group regardless of whether it sintered ($p < 0.05$). The level of phospho-JNK protein in non-sintered HA was expressed about twice as much as that of sintered HA. Non-sintered β-TCP also showed higher protein expression than sintered β-TCP. An inhibitor suppressed JNK signaling by calcium phosphates (Fig. 14A).

3.3.4 Effect of sintering on the Src signaling pathway

HA and β-TCP significantly suppressed the Src signaling pathway regardless of whether it sintered ($p < 0.05$). However, the level of phospho-Src protein was slightly increased in non-sintered HA compared to sintered HA. However, the difference was insignificant (Fig. 14B).

3.3.5 Effect of sintering on the EGFR signaling pathway

The amount of total EGFR protein did not change with sintered or non-sintered HA and β-TCP. However, the activated phospho-EGFR showed different patterns. Sintered HA and β-TCP increased the phospho-EGFR protein to the control level, whereas non-sintered HA and β-TCP significantly suppressed the phospho-EGFR protein level ($p < 0.05$). Compared to non-sintered, the EGFR signaling pathway for sintered calcium phosphates was approximately five times higher (Fig. 14C).

3.3.6 Effect of sintering on the Raptor/mTORC1 signaling pathway

The effect of the sintered calcium phosphates on the Raptor signal path was confirmed. As a result, the total Raptor protein level was not affected by the sintering of calcium phosphates (Fig. 14D).

3.3.7 Effect of sintering on the p38 signaling pathway

The effect of the sintering of calcium phosphates on the p38 signaling pathway was evaluated. The p38 signaling pathway was significantly activated in all experimental groups compared to the control group ($p < 0.05$). The total p38 protein was not affected by the sintering of calcium phosphates and was similar to the control group. HA did not affect the expression of the phospho-p38 protein with or without sintering, whereas, in the case of β-TCP, the sintered β-TCP decreased in protein expression compared to non-sintered β-TCP (Fig. 15A).

3.3.8 Effect of sintering on the Akt signaling pathway

Total Akt protein expression was significantly decreased in sintered or non-sintered HA and β-TCP compared to the control group ($p < 0.05$). HA increased total Akt protein expression due to sintering compared to non-sintered HA. However, phospho-Akt S473 protein expression was hardly affected by sintering. β-TCP had similar total Akt protein expression regardless of sintering, while phospho-Akt S473 protein expression decreased during sintering (Fig. 15B).

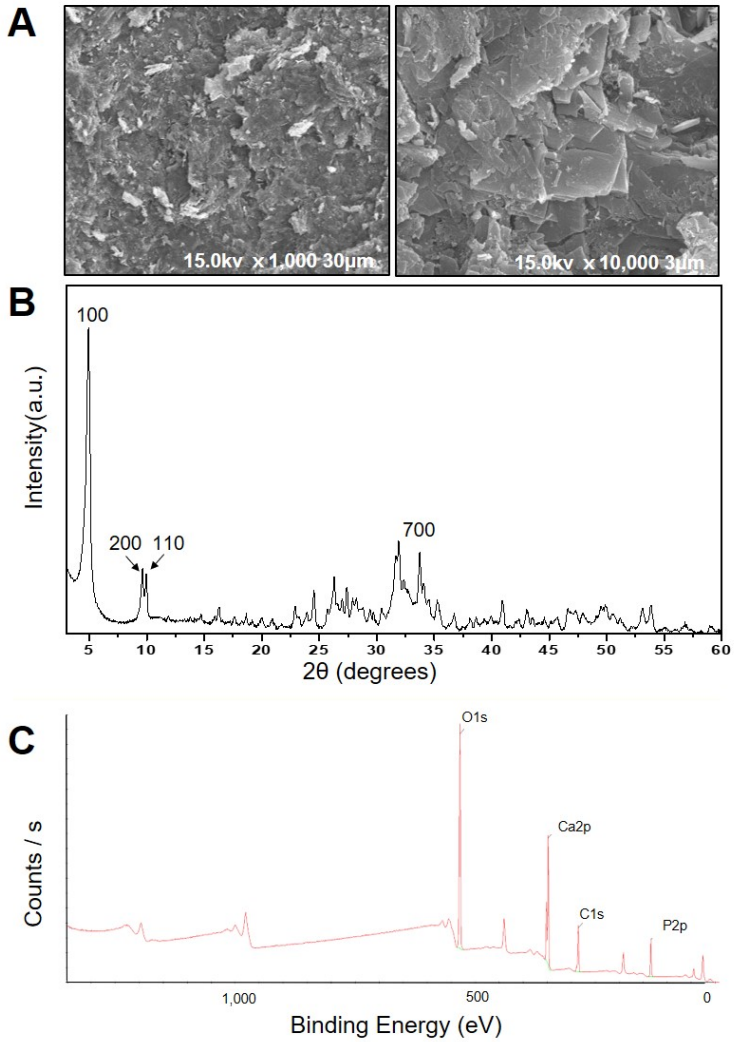

Fig. 1. Characteristics of OCP. (A) SEM images (×1,000 ×10,000) (B) XRD pattern (C) XPS pattern. (Reproduced with permission from Jung, Y.A.; Kim, J.S.; Kim, S.K.; Chung, S.H.; Wie, J.H. *Int J Med Sci* **2022**, 19(12):1724-1731. Copyright 2022 Ivyspring International Publisher)

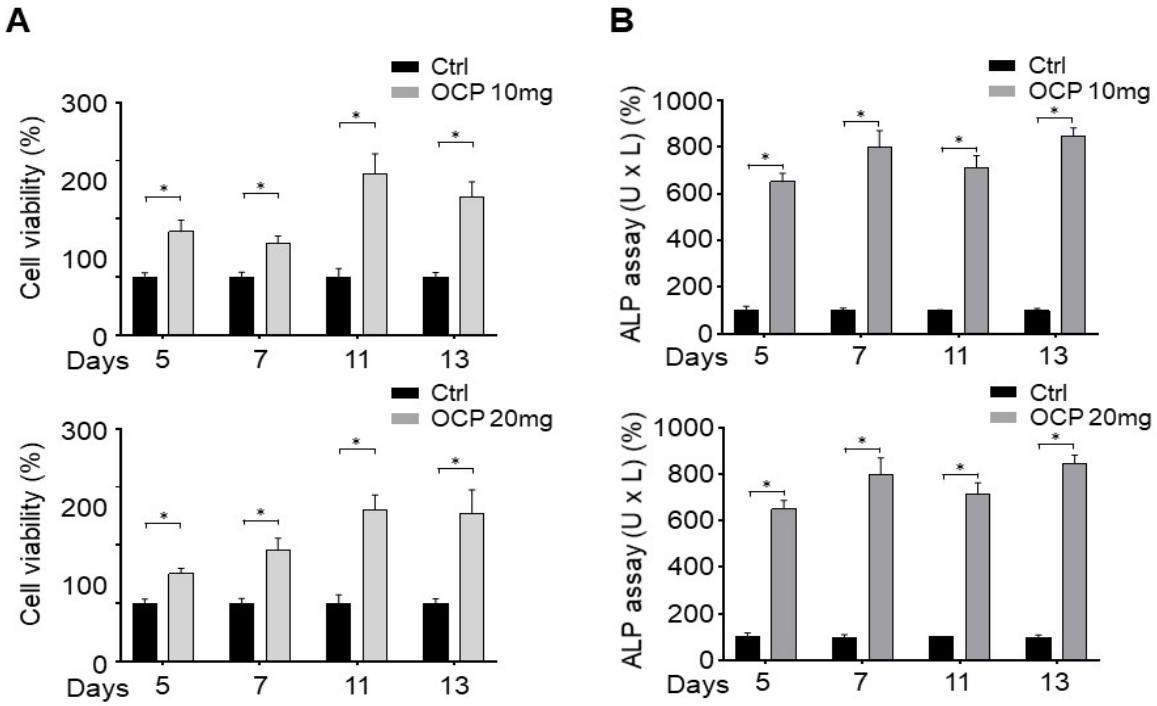

Fig. 2. Results on the effect of OCP on cell proliferation and biomineralization. (A) MTT assay (B) ALP activity of MG63 cells was measured with or without OCP (10 mg/mL and 20 mg/mL). Data are means ± SEM. *$p < 0.05$, n = 3, student's t-test. (Reproduced with permission from Jung, Y.A.; Kim, J.S.; Kim, S.K.; Chung, S.H.; Wie, J.H. *Int J Med Sci* **2022**, 19(12):1724-1731. Copyright 2022 Ivyspring International Publisher)

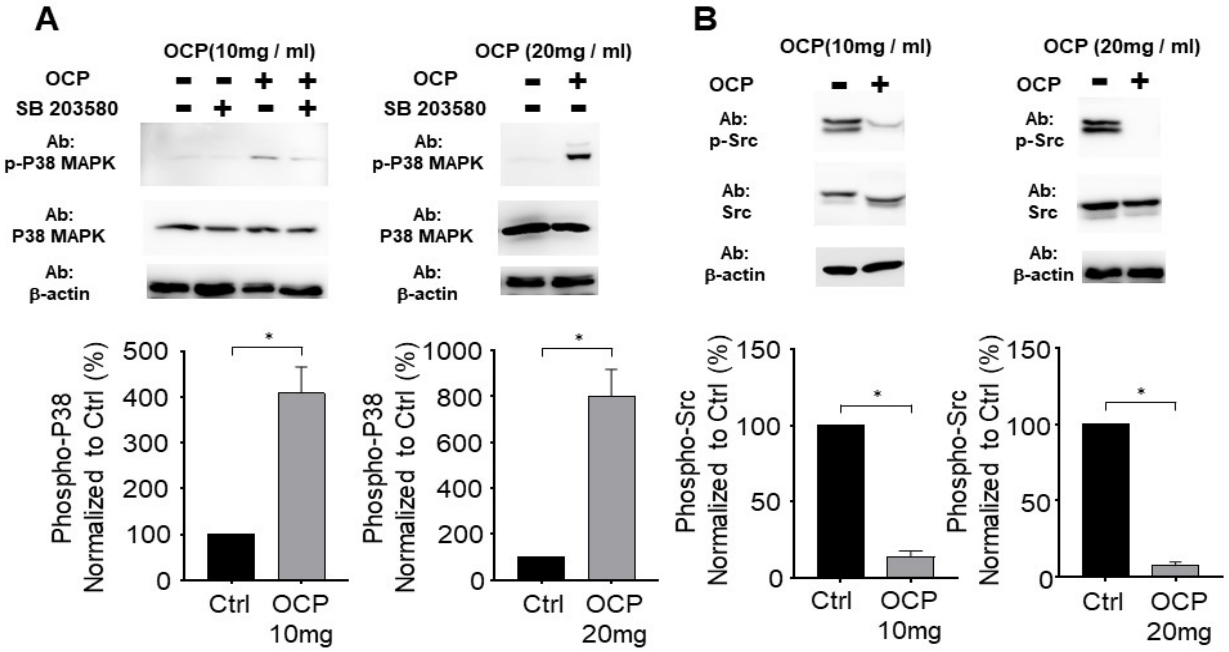

Fig. 3. Results on the effect of OCP on the p38 and Src signaling pathway. (A) p38 pathway (B) Src pathway. The bottom graph shows results normalized to the control. Data are means ± SEM. *$p < 0.05$, student's t-test. (Reproduced with permission from Jung, Y.A.; Kim, J.S.; Kim, S.K.; Chung, S.H.; Wie, J.H. *Int J Med Sci* **2022**, 19(12):1724-1731. Copyright 2022 Ivyspring International Publisher)

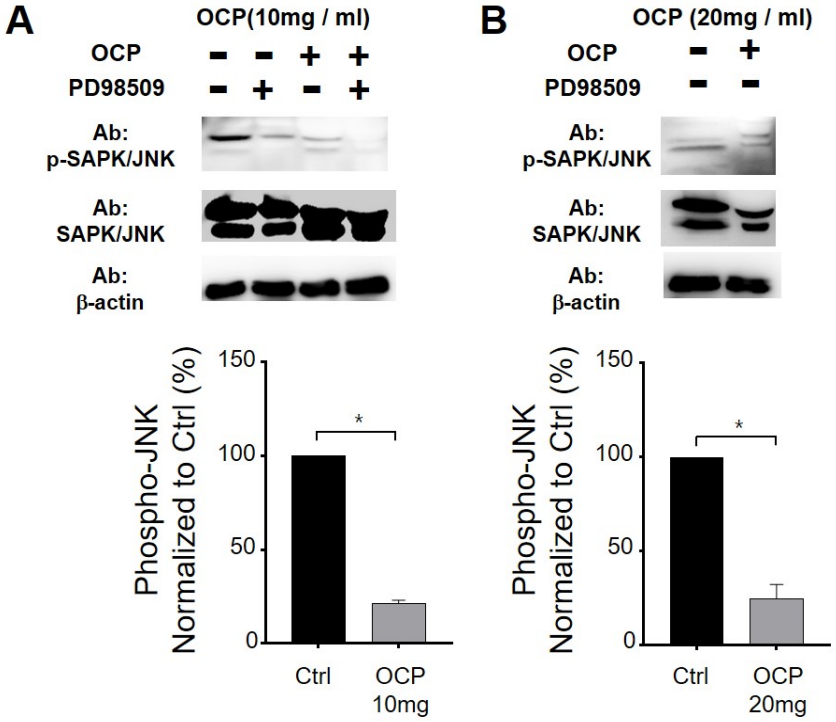

Fig. 4. Results on the effect of OCP on the JNK signaling pathway.

(A) 10mg/mL (B) 20mg/mL of OCP treatment. The bottom graph shows results normalized to the control. Data are means ± SEM. *$p < 0.05$, student's t-test. (Reproduced with permission from Jung, Y.A.; Kim, J.S.; Kim, S.K.; Chung, S.H.; Wie, J.H. *Int J Med Sci* **2022**, 19(12):1724-1731. Copyright 2022 Ivyspring International Publisher)

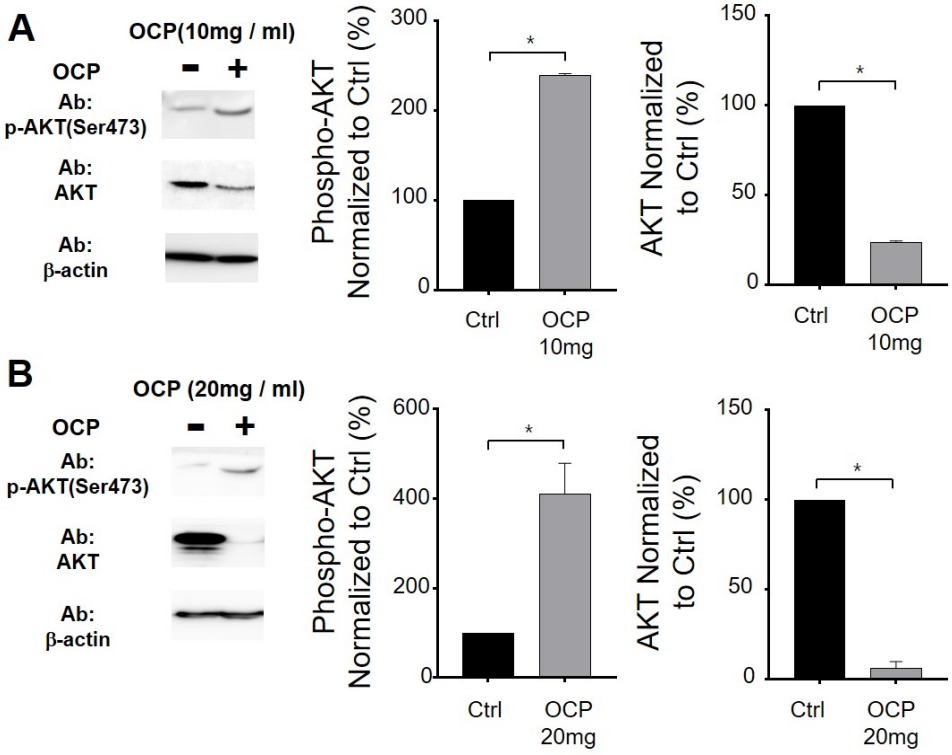

Fig. 5. Results on the effect of OCP on the Akt signaling pathway.

(A) 10 mg/mL (B) 20 mg/mL of OCP treatment. The bottom graph shows results normalized to the control. Data are means ± SEM. *$p < 0.05$, student's t-test. (Reproduced with permission from Jung, Y.A.; Kim, J.S.; Kim, S.K.; Chung, S.H.; Wie, J.H. *Int J Med Sci* **2022**, 19(12):1724-1731. Copyright 2022 Ivyspring International Publisher)

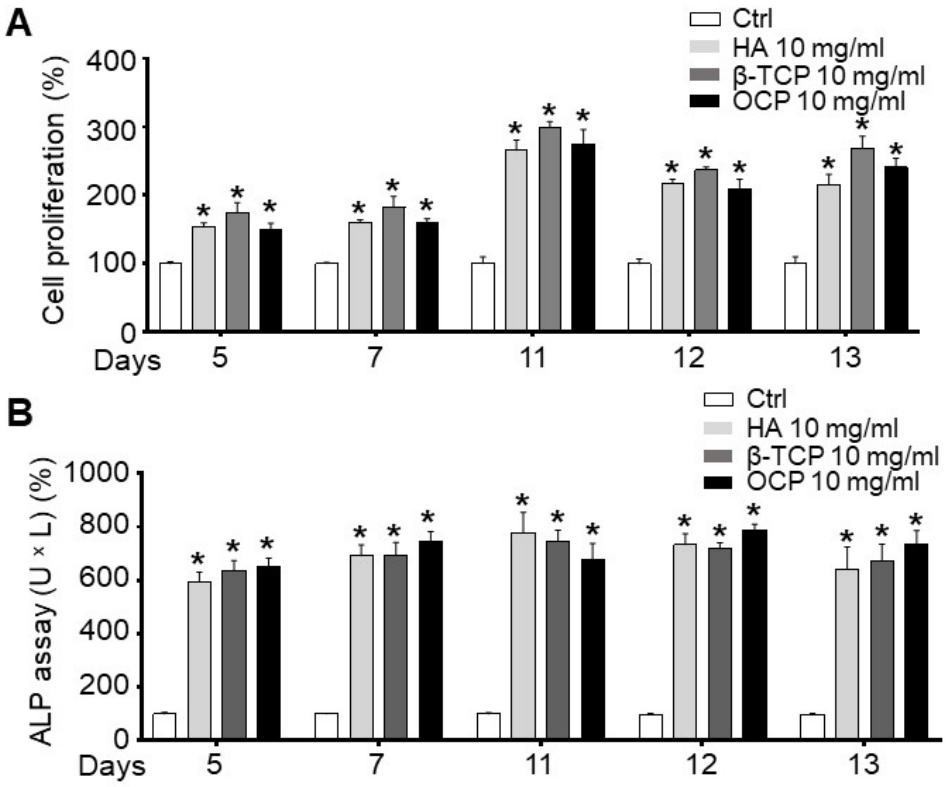

Fig. 6. Results on the effect of calcium phosphates on cell proliferation and biomineralization. (A) MTT assay (B) ALP assay. Data are means ± SEM. *$p < 0.05$, unpaired two-tailed t-test.

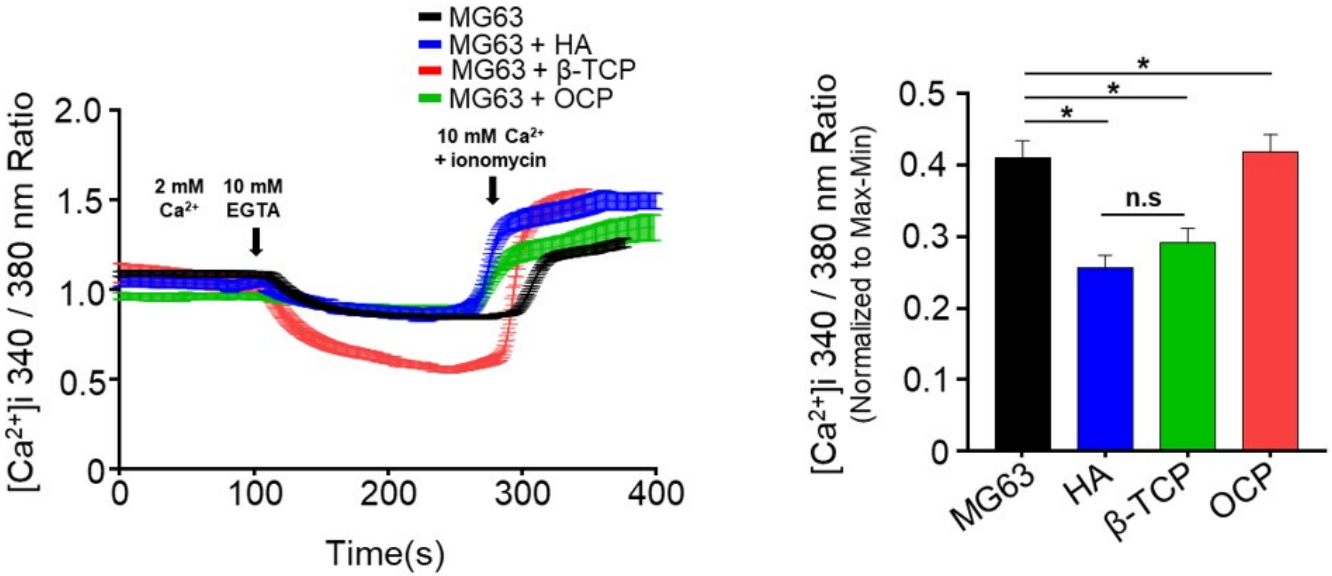

Fig. 7. Results on the effect of calcium phosphates on the intracellular Ca^{2+} levels. The bars graph indicated that normalized into the maximum (10 mM Ca^{2+}, 5 μM ionomycin) and minimum value (10 mM EGTA). Data are expressed as means ± S.E.M. n.s: not significant, *$p < 0.05$, unpaired two-tailed t-test

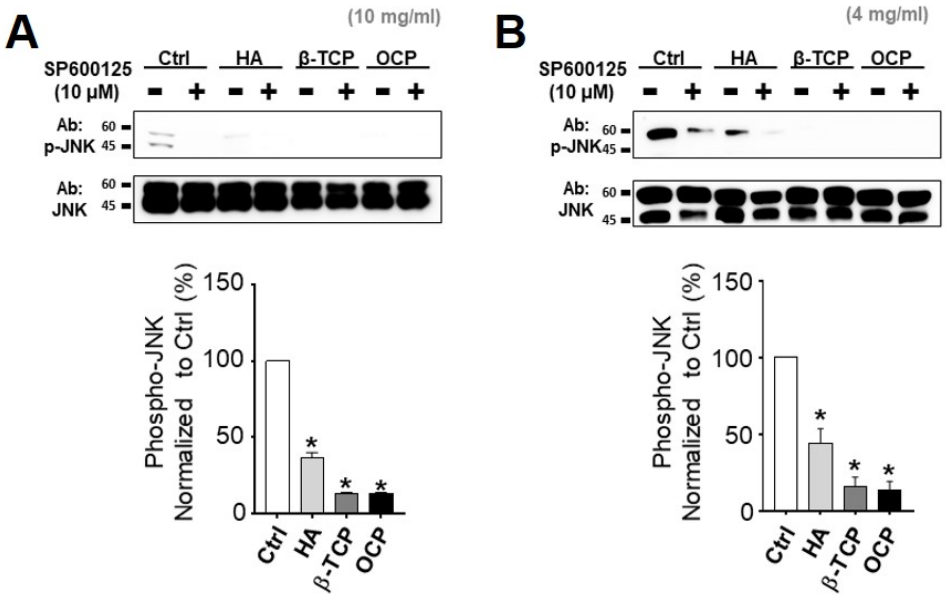

Fig. 8. Results on the effect of calcium phosphates on the p38 signaling pathway. (A) 10 mg/mL (B) 4 mg/mL of calcium phosphates treatment. The bar graphs show the means ± SEM. * $p < 0.05$, unpaired two-tailed t-test.

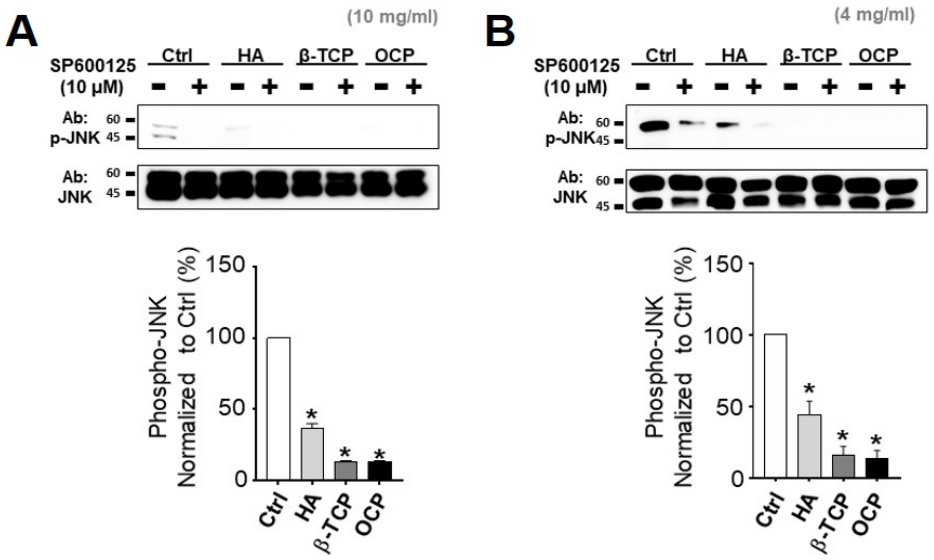

Fig. 9. Results on the effect of calcium phosphates on the JNK signaling pathway. (A) 10 mg/mL (B) 4 mg/mL of calcium phosphates treatment. The bar graphs show the means ± SEM. * $p < 0.05$, unpaired two-tailed t-test.

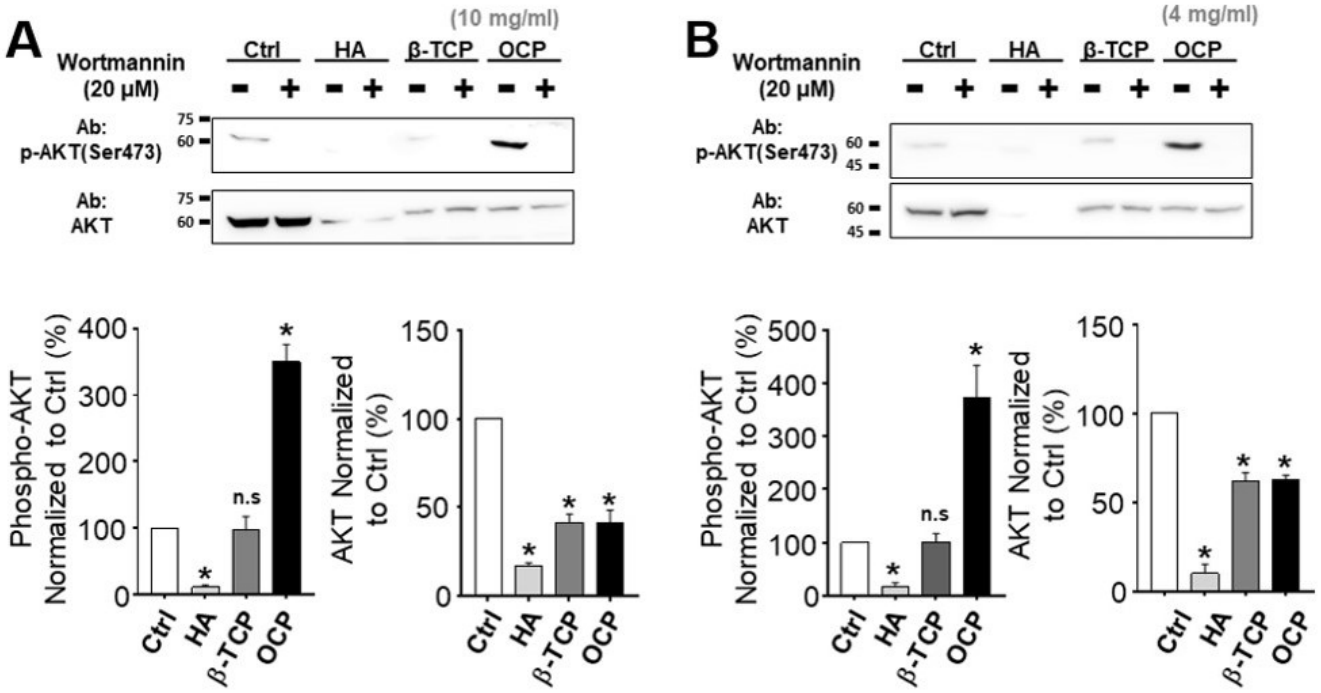

Fig. 10. Results on the effect of calcium phosphates on the Akt signaling pathway. (A) 10 mg/mL (B) 4 mg/mL of calcium phosphates treatment. The bar graphs show the means ± SEM. * $p < 0.05$, unpaired two-tailed t-test.

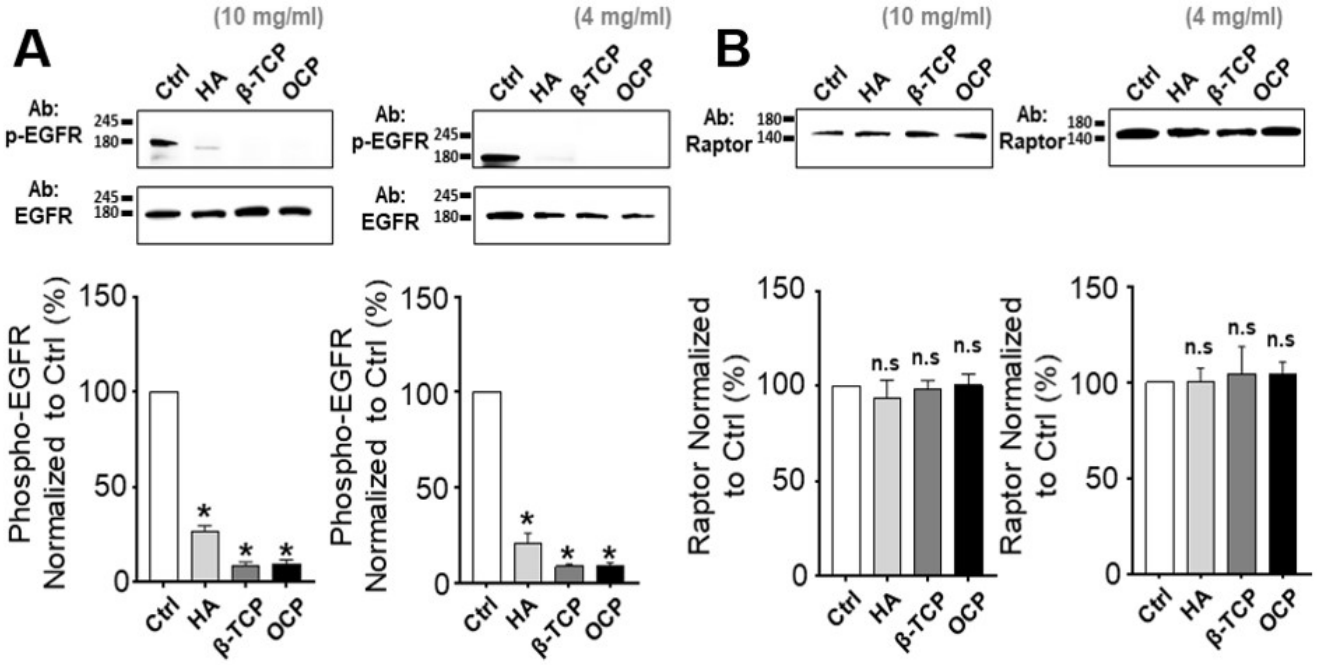

Fig. 11. Results on the effect of calcium phosphates on the EGFR and Raptor pathway. (A) EGFR pathway (B) Raptor pathway. The bar graphs show the means ± SEM. * $p < 0.05$, unpaired two-tailed t-test.

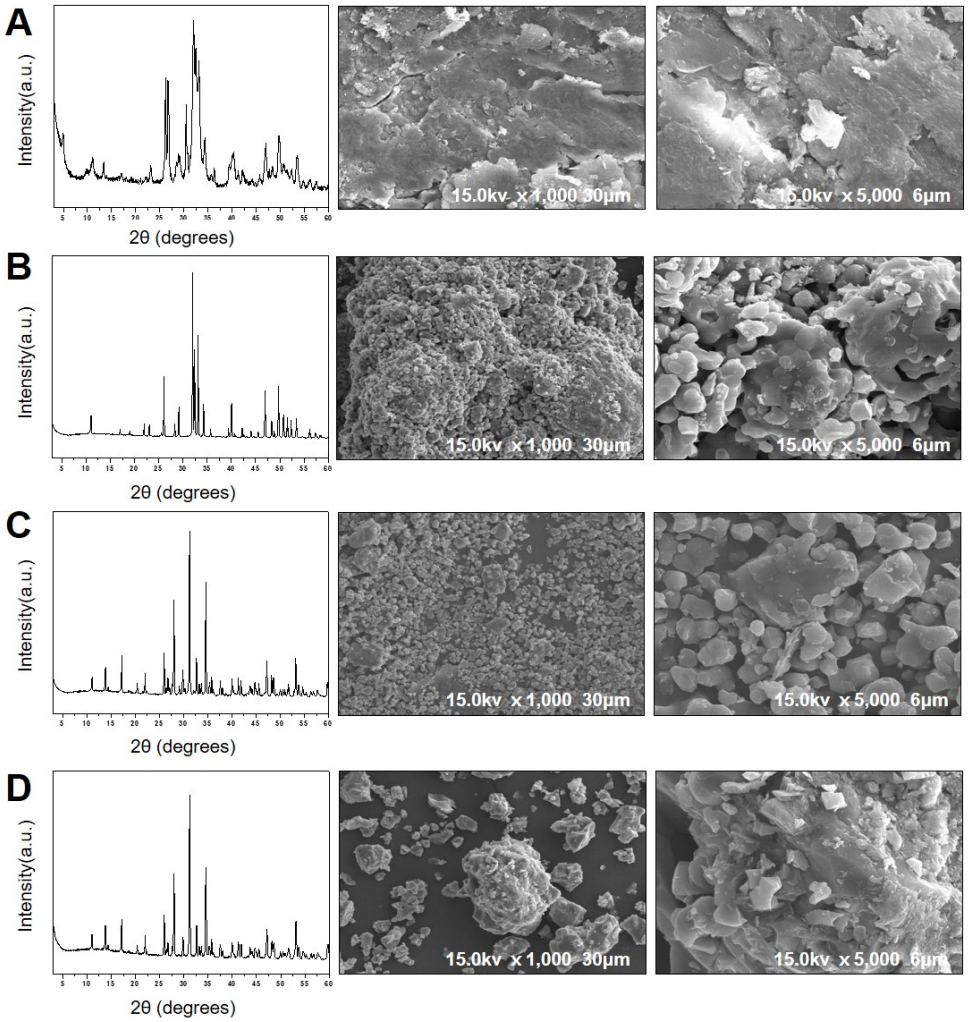

Fig. 12. Characteristics of sintered and non-sintered HA and β-TCP. XRD pattern and SEM images (left: x1,000, right: x5,000). (A) non-sintered HA (B) sintered HA (C) non-sintered β-TCP (D) sintered β-TCP.

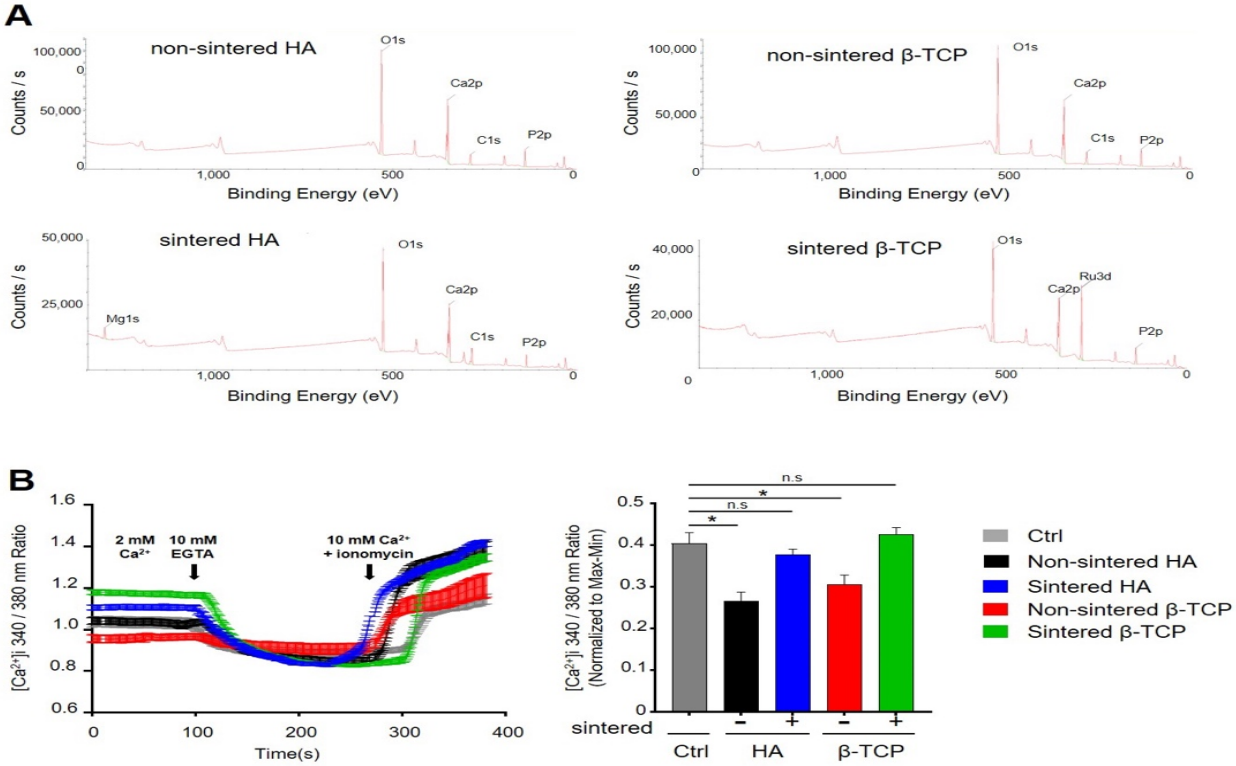

Fig. 13. XPS patterns and intracellular Ca^{2+} levels of sintered and non-sintered HA, β-TCP. (A) XPS (B) intracellular Ca^{2+} levels. The bar graph indicates values normalized to those of the Ctrl; values are presented as mean ± SEM. * $p < 0.05$; n.s: not significant. One-way ANOVA test

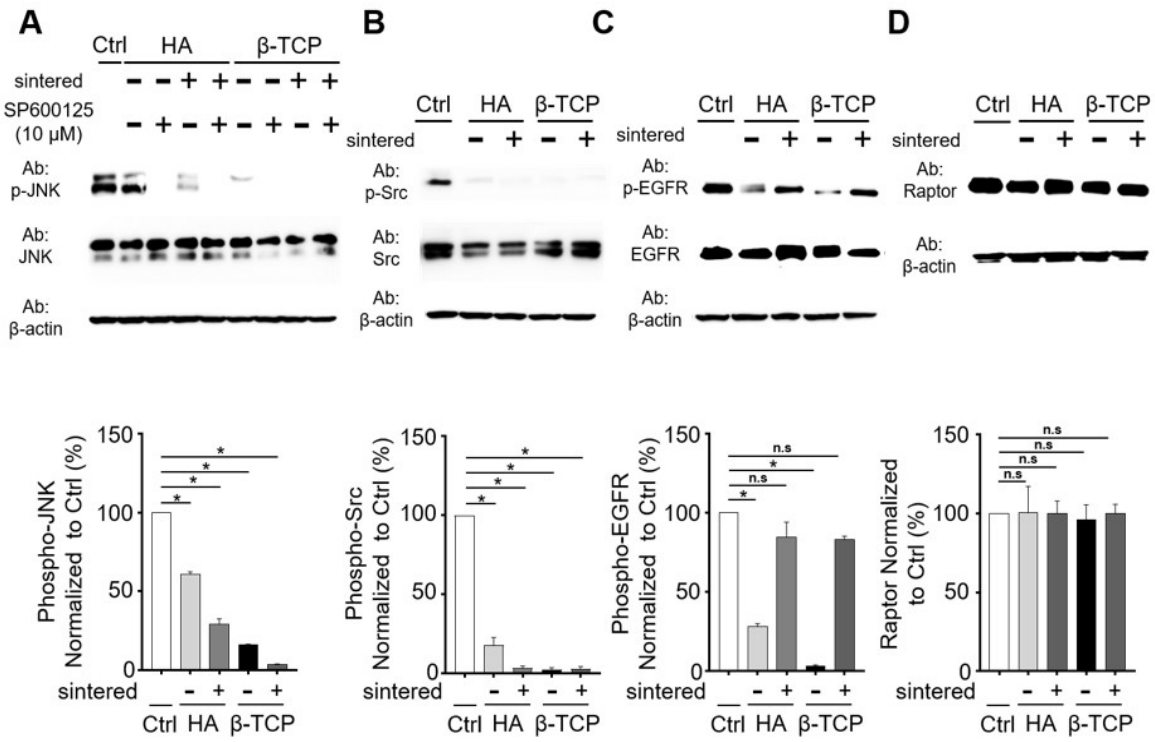

Fig. 14. Results on the effect of sintering on various intracellular signaling pathways. (A) JNK pathway (B) Src pathway (C) EGFR pathway (D) Raptor pathway. The bar graph indicates values normalized to those of the Ctrl, and values are mean ± SEM. *$p < 0.05$; n.s: not significant. One-way ANOVA test.

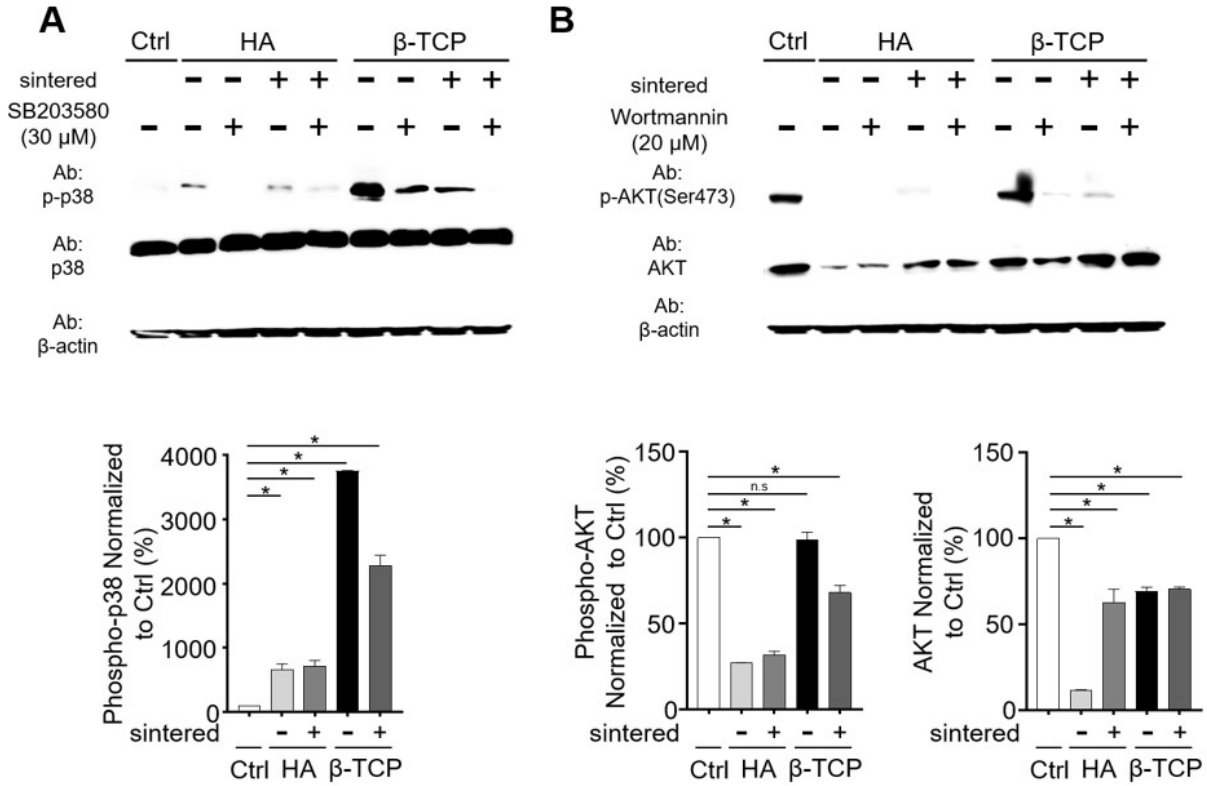

Fig. 15. Results on the effect of sintering on p38 and Akt signaling pathways. (A) p38 pathway (B) Akt pathway. The bar graph indicates values are presented as mean ± SEM. *$p < 0.05$; n.s: not significant. One-way ANOVA tes

4. Discussion

4.1 Influence of OCP on various cell signaling pathways

This study demonstrated that high-purity OCP regulates cellular function mechanisms. In the past, OCP research focused on physicochemical changes or wound healing and bone formation. However, studies on why OCP increases intracellular signaling pathways have yet to be published. It was confirmed that OCP is a factor that increases the viability and proliferation of osteoblasts.

The Ca/P ratio of the OCP used in this study was 1.36, slightly higher than the stoichiometric ratio. Previous studies have shown that OCP with a slightly higher Ca/P ratio of 1.37 than the stoichiometric ratio of 1.33 in biomaterials showed bone formation and higher bioactivity. It is thought that this part may have contributed to osteoblast proliferation[75,84].

However, Ca/P measured by XPS is 1.23, which is different from the result of SEM-EDS. In general, SEM-EDS is measured at a depth of 1 μm where a minute part of the sample surface is analyzed for material information to know about the bulk component. In contrast, XPS is mainly based on information on the oxide layer, hydroxide layer, and adsorption layer (Fig. 1).

OCP increased cell proliferation more than twice after 11 days (Fig. 2A), confirming that OCP is a factor that increases the viability and proliferation of osteoblasts. In addition, results of ALP activity demonstrated that OCP acts by upregulating biomineralization in osteoblasts (Fig. 2B). According to this result, OCP regulated the complex process of cell proliferation.

First, mitogen-activated protein kinase (MAPK) controls several crucial transcription factors that influence the differentiation and operation of osteoblasts and osteoclasts[85]. p38 is a member of the MAPK family of extracellular stimuli-processing enzymes. They have a Ser/Thr kinase domain activated when another Ser/Thr kinase phosphorylates it. Therefore, p38/MAPK phosphorylation affected cell differentiation and proliferation.

According to this experiment, OCP treatment increased the phosphorylation of p38/MAPK in cells as opposed to non-cells. SB203580, a p38/MAPK inhibitor, decreased p38/MAPK phosphorylation but did not influence basal protein level (Fig. 3A). Previous study reported that OCP reportedly increased p38 phosphorylation in other cells[86], assisted in activating the p38 signaling pathway and decrease the intracellular Ca^{2+} level. OCP is a type of calcium phosphate formed with a calcium-to-Pi ratio of 1.36. The implanted OCP is hydrolyzed to CDHA, absorbing the surrounding calcium and releasing a small amount of Pi. It also promotes osteoblast proliferation by increasing ALP and osterix upregulation factors of osteoblast differentiation[87].

Second, tyrosine kinases from the Src family modulate various cellular signaling pathways, including cell proliferation, survival, morphology, and cytoskeletal remodeling[88]. Src activity is crucial for preserving the homeostasis of the bones. Additionally, this signal regulates growth, invasion, and metastasis[89].

Src also blocks the function of RUNX2, a key regulator of osteoblast differentiation, which reduces osteoblasts' ability to generate bone[90]. Previous studies have reported that the Src signaling pathway inhibits osteoblast differentiation[121]. Although Src phosphorylation is recognized as a crucial regulatory route, the underlying mechanisms are still poorly understood. In the present study, Src phosphorylation signaling was interrupted by OCP in MG63 cells (Fig. 3B).

Third, JNK (programmed cell death) plays a vital role in various intracellular biological processes, such as the immune response, tissue homeostasis, and regular cell turnover[91]. In MG63 cells, OCP treatment suppressed JNK phosphorylation. On the addition of the PD98509 inhibitor, JNK phosphorylation in MG63 cells with or without OCP also decreased (Fig. 4).

Fourth, Akt is essential for several interconnected signaling processes that affect cell growth, metabolism, and death[92]. Cancer, diabetes, cardiovascular disease, and neurological conditions are all linked to Akt signaling abnormalities. Cell proliferation is promoted by Akt phosphorylation. In this study[93], the Akt expression level of the OCP-treated

cells was decreased compared to the control. Interestingly, OCP strongly encouraged the phosphorylation of Akt. Therefore, OCP is a factor that promotes Akt phosphorylation, which stimulates cell proliferation (Fig. 5). However, with a decrease in total Akt protein, further studies are needed to understand how OCP promotes Akt phosphorylation. Previous studies have reported that high concentrations of salinomycin (a cancer chemotherapeutic agent) decrease total Akt protein and increase Akt phosphorylation[94]. One possibility is that calcium and phosphate released during conversion to CDHA when OCP is hydrolyzed speculated to be the factors driving changes in the Akt signaling pathway. However, the specific mechanism is still unclear, and further studies are needed.

4.2 Regulation of intracellular multiple pathways by three calcium phosphates

This study confirmed that three types of calcium phosphates mediate intracellular signaling pathways. HA, β-TCP and OCP stimulated cellular proliferation and ALP activity, demonstrating the ability of calcium phosphates to facilitate bone regeneration[95] (Fig. 6).

OCP treatment did not affect the intracellular Ca^{2+} level. However, HA and β-TCP decreased the intracellular Ca^{2+} levels compared to the control (Fig. 7). In a previous study, the Ca^{2+} concentration of OCP was relatively lower than HA, β-TCP after immersion in culture media. The low Ca^{2+} concentration of OCP inhibited the initial adhesion of osteoblast ST-2 cells. HA and β-TCP showed superior initial cell proliferation compared to OCP. However, after about 21 days, cell proliferation of the three types of calcium phosphates had similar levels[41]. Previous in vivo studies have demonstrated no difference in promoting new bone formation between calcium phosphates with high and low solubility. There was no difficulty stimulating new bone formation even when calcium phosphates have low solubility, and calcium levels are low[42]. ALP was maximal when the extracellular Ca^{2+} concentration was 1.2 to 1.8

mM. The range of change in the local concentration of extracellular calcium that can regulate osteoblast activity is estimated to be much smaller than 8 to 40 mM. It is expected to be similar to the sensitivity of parathyroid extracellular calcium-sensing receptor[96]. When the extracellular Ca^{2+} concentration is increased to a level higher than the cell sensitivity range, ALP activity and release are inhibited[97].

Numerous fundamental cellular functions, such as cellular motility, differentiation, and proliferation, are regulated by intracellular calcium signaling. In bone cells, strain, pressure, and fluid flow rapidly activate an extracellular influx and intracellular release[98]. The source of Ca^{2+} seems to be intracellular stores following the activation of phospholipase C (PLC) and IP3 signaling[99], and inhibiting the release of Ca^{2+} reduces the mechanically induced upregulation of osteogenic gene expression[100] and eliminates the load-induced formation of bone in vivo[101]. Mechanical stimulation-induced intracellular calcium mobilization also controls downstream signaling, such as protein kinase A (PKA), MAPK, c-Fos (related to neuronal activity), and nuclear translocation of NF-κB[102].

Previous studies have shown that OCP helps activate the p38 signaling pathway in ST-2 cells by regulating intracellular Ca^{2+} concentration[86]. In a previous study, when mechanical stimulation was applied to MC3T3-E1 osteoblasts, the intracellular Ca^{2+} concentration increased, and the Akt S473 was activated to suppress apoptosis[26]. The intracellular Ca^{2+} level of OCP was higher than other calcium phosphates. In addition, compared to other calcium phosphates, OCP significantly activated Akt S473.

These findings indicate that OCP acts as a mechanical stimulus in osteoblasts to protect osteoblasts from apoptosis. The effects of three calcium phosphates on p38, JNK, EGFR, and Akt signaling pathways essential for cell proliferation were confirmed. In this experiment, treatment with three calcium phosphates increased p38 phosphorylation, followed by SB203580 inhibiting p38 phosphorylation. Among them, β-TCP showed the most prominent protein expression. However, 4 mg/mL HA had little effect on p38 phosphorylation (Fig. 8).

This study confirms that β-TCP and OCP sharply decreased JNK phosphorylation, involved in programmed cell death and various biological functions, including tissue homeostasis and average cell turnover. In contrast, HA treatment had a relatively weak effect on JNK phosphorylation (Fig. 9).

The EGFR signaling pathway has a significant role in several carcinomas[103]. The experimental results indicated that β-TCP, OCP, and HA significantly decreased phospho-EGFR levels in MG63 cells. In contrast, overall EGFR levels were unaffected by the types of calcium phosphates (Fig. 11A).

Finally, HA decreased the total Akt protein levels and Akt phosphorylation, whereas β-TCP had a weak effect on Akt phosphorylation but reduced the total Akt levels. However, OCP markedly increased Akt phosphorylation by more than thrice while reducing the total Akt protein levels by almost half (Fig. 10). Previously, anti-cancer drugs have been demonstrated to increase Akt phosphorylation despite decreased total Akt protein level[94]. This means that cell proliferation and signaling pathways may show different patterns depending on the properties of the calcium phosphates, and further research is needed.

4.3 Effects of sintering on intracellular multiple signaling pathways

Sintering is a thermal process that converts particles into a constant solid mass by heat and/or pressure without melting particles. It is applied to impart the strength and integrity of calcium phosphates[104]. Sintering the calcium phosphate at a high temperature for a long time is known to form a secondary phase due to thermal instability. Excessive particle growth can hinder the densification process[105]. The material undergoes various physicochemical deformation during sintering[106].

Several previous studies on existing material properties, cellular wound

healing, and ALP activity have mainly used sintered calcium phosphates. It remains unknown how sintering changes the intracellular signaling pathways. Thus, this study demonstrated that calcium phosphates had different effects on cell signaling pathways with or without sintering treatment.

XRD analysis confirms that β-TCP did not change regardless of whether it was sintered. However, changes in the pattern of HA were observed after sintering. Additionally, the SEM image showed that the HA was different after sintering (Fig. 12A, 12B). XPS analysis showed that Mg1s were present in the sintered HA but not in the non-sintered HA. The sintered β-TCP contained Ru3d, and other ions were identical to the non-sintered β-TCP (Fig. 13A).

Compared to non-sintered biomaterials, sintered biomaterials have higher intracellular Ca^{2+} levels (Fig. 13B). Previous studies have reported that activation of EGFR generates a Ca^{2+} signal, leading to a transient increase in intracellular Ca^{2+} level. The rapidly increased cytoplasmic Ca^{2+} levels return to basal levels within minutes[107]. EGFR signaling pathway, a characteristic of several carcinomas, extensively impacts the intracellular signaling pathways[103]. The EGFR signaling pathway was found to be significantly altered by sintering in the experimental results of this study.

The intracellular Ca^{2+} levels of the sintered calcium phosphates were higher than that of the non-sintered calcium phosphates. The calcium phosphates with sintering dramatically increased phospho-EGFR protein levels in MG63 cells. In contrast, non-sintered calcium phosphates had no impact, and total protein levels remained unchanged (Fig. 14C). These results indicate that the activation of the EGFR signaling pathway due to sintering is associated with an increase in intracellular Ca^{2+} level.

As a result of the JNK signaling pathway reported to regulate cell death and cell processes related to cell hemostasis and immune response[91], all kinds of calcium phosphate showed a decrease compared to the control group, but non-sintered calcium phosphate showed an increase compared to sintered calcium phosphate through high phospho-JNK protein expression. In the sintered β-TCP negligible phosphorylated JNK protein was detected (Fig. 14A). These results indicate that the JNK signaling pathway is affected by

both sintering and the type of calcium phosphate.

According to previous research, Src activity regulates invasion and growth and upholds bone homeostasis. Tyrosine kinases, a member of this family, impact cell morphology, survival, and proliferation[88]. Previous research reported that Src inhibited osteoblast proliferation, explaining cell proliferation[108]. This study discovered that calcium phosphates reduced the levels of phospho-Src protein both with and without sintering (Fig. 14B). Total Raptor/mTORC1 protein levels were not affected by types of calcium phosphates and sintering (Fig. 14D).

p38 is a member of the MAPK family, a signaling pathway that promotes cell differentiation and proliferation[85]. Effects of intracellular signaling pathways caused by sintering were found to be different depending on the type of calcium phosphate. In HA, phospho-p38 protein expression was observed at a low level and there was no effect of sintering. On the other hand, β-TCP showed a significant difference in p38 protein expression by sintering. Non-sintered β-TCP dramatically increased phospho-p38 protein levels, whereas sintered β-TCP reduced protein expression (Fig. 15A). Thus, sintering appears to be a factor that slightly inhibits the activity of the p38 signaling pathway of β-TCP. In the AKT signaling pathway, calcium phosphate had lower protein expression than the control group. In comparison to sintered β-TCP, non-sintered β-TCP significantly increased Akt phosphorylation level. Non-sintered β-TCP increased Akt phosphorylation more than twice as much as sintered β-TCP. However, HA did not impact the phosphor-Akt level and decreased total Akt protein levels both with and without sintering. Both wortmannin and the Akt inhibitor inhibited all Akt signaling pathways. Interestingly, total protein levels show that non-sintered HA significantly decreased them by more than five times compared to other calcium phosphates (Fig. 15B). These findings suggest that each type of calcium phosphate individually influences the cell signaling pathway, and sintering has a significant impact. However, the specific mechanism is unknown and needs more research.

5. Conclusion

Calcium phosphates are excellent biocompatible materials used for various purposes for bone regeneration. Numerous studies have shown that calcium phosphates promote the proliferation of osteoblasts and activate differentiation factors. However, until now, there has been no study on changes in intracellular signaling pathways and intracellular Ca^{2+} levels caused by calcium phosphates. Therefore, in this study, the mechanism of the intracellular signaling pathway and Ca^{2+} levels change by calcium phosphates were identified through a physiological approach. Unlike the existing consequential approach, this study observed intracellular changes in the process of osteoblastic cell proliferation, which is different from previous studies.

The regulation mechanism of intracellular signaling pathways involved in cell proliferation and the influence of Ca^{2+} levels in cells differed depending on the type of calcium phosphate with other physicochemical properties. These findings imply that while calcium phosphates with different physicochemical properties eventually exhibit similar levels of osteoblast cell proliferation, intracellular signaling pathways can display different patterns, and their physicochemical properties can act as regulators for specific signaling pathways in cells. F

Further research is needed to determine which physicochemical properties of calcium phosphates affect intracellular signaling pathways and intracellular Ca^{2+} levels. In addition, more research is needed on whether the influence of calcium phosphates in various cells occurs in similar patterns by checking whether there is a difference in signal transmission paths according to cell types.

The results can be used as primary data to predict intracellular signaling pathways using calcium phosphates. Moreover, it can be used as a basis for research that maximizes material effects by regulating specific intracellular signaling pathways using drugs when applying calcium phosphates.

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국문초록

인산칼슘이 인간 조골세포의 세포 내 다중 신호전달 경로에 미치는 영향

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목 적:

본 연구는 인산칼슘이 인간 조골세포 증식과 관련된 세포 내 다중 신호 전달 경로를 조절하는 효과를 규명하는 것을 목적으로 한다. 이에 본 연구는 3종의 인산칼슘이 MG63 세포의 증식에 관여하는 세포 내 다중 신호전달 경로에 미치는 영향을 조사하고, 소결로 인한 인산칼슘의 물리화학적 변화가 세포 내 칼슘 농도 및 세포 내 다중 신호전달 경로에 미치는 영향에 관해 연구하였다.

재료 및 방법:

인산칼슘으로 인한 세포 내 다중 신호전달 경로의 변화를 확인하기 위해 HA, β-TCP, OCP를 이용하였다. 소결로 인한 인산칼슘의 물리화학적 특성 변화가 세포 내 미치는 영향에 관한 연구는 소결 전후의 HA, β-TCP를 사용하였다. 인산칼슘의 형태는 세포와 접촉 및 이온 방출을 최대화하기 위해 분말 형태의 인산칼슘을 이용하였다.

인산칼슘의 물리화학적 특성은 XRD, SEM, XPS 분석을 통해

확인하였다. 세포 증식과 biomineralization 활성은 MTT와 ALP 분석으로 확인하였다. 세포 내 다중 신호 전달 경로는 western blotting 분석을 통해 개별 신호전달 경로의 단백질을 검출하여 확인하였다. 세포 내 Ca^{2+}은 인산칼슘이 처리된 MG63 세포와 처리되지 않은 MG63 세포를 형광 염료인 Fura-2 AM과 결합시켜 고속 파장 변환 장치와 도립현미경을 이용하여 측정하였다.

결 과:

OCP는 p38 신호 경로를 촉진하고 면역 반응에 관여하는 JNK 신호 경로를 억제하였다. 조골세포 분화인자 RUNX2를 차단하는 것으로 알려진 Src 신호 경로는 OCP에 의해 억제되었다. 특히, 세포 성장과 대사에 필수적인 역할을 하는 Akt 신호전달 경로는 OCP에 의해 효율적으로 활성화되었다.

3종의 인산칼슘은 조골세포 증식과 ALP 활성을 대조군에 비해 통계적으로 유의하게 증가시켰다. 그러나 세포 내 다중 신호전달 경로는 인산칼슘의 종류에 따라 활성화되는 신호전달 경로가 상이하였다. 세포 내 칼슘은 HA 및 β-TCP가 세포 내 칼슘을 대조군에 비해 다소 감소시켰지만, OCP는 대조군과 유사한 수치를 보였다.

소결에 의한 인산칼슘의 물리화학적 특성 변화는 세포 내 칼슘을 통계적으로 유의하게 증가시켰다. 그러나, 세포 내 신호 경로는 개별 경로마다 소결에 의한 영향이 상이하였고, 인산칼슘의 종류와 소결의 영향이 혼재된 양상으로 나타났다.

결 론:

본 연구의 결과를 통해 상이한 물리화학적 특성을 갖는 3종의 인산칼슘은 결과적으로는 유사한 수준의 조골세포 증식을 유도하지만, 인산칼슘의 종류에 따라 상이한 세포 내 신호 경로를

활성화한다는 것을 확인할 수 있었다. 특히 소결로 인한 영향은 세포 내 특정 신호 경로에서만 관찰되었고, 그 패턴은 인산칼슘 종류에 따라 상이하였다. 이러한 결과는 인산칼슘의 물리화학적 특성이 특정 신호전달 경로를 조절하는 인자로 작용할 수 있음을 시사하며, 향후 인산칼슘의 다양한 물리화학적 특성을 개별적으로 조절하여 세포 내 신호전달 경로에 영향을 미치는 인산칼슘의 특정 인자를 규명하기 위한 심도 있는 연구가 필요하다.

본 연구의 결과는 향후 인산칼슘의 물리화학적 변화에 따른 세포 내 신호전달 경로의 변화를 예측하기 위한 1차 자료로 활용될 수 있으며, 또한 인산칼슘을 활용한 다양한 연구에서 약물과 함께 특정 세포 내 신호전달 경로를 조절하여 골재생 효과를 촉진하는 연구의 1차 자료로 활용될 수 있을 것으로 사료된다.

주요어: 수산화인회석, β-삼인산칼슘, 인산팔칼슘, 인산칼슘, 세포 내 신호 전달 경로, 세포 내 칼슘

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