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Osteoconductivity and Mineralization of Different Commercial Bone Substitute Materials and Newly Hybrid Bone Substitute Material Between Xenograft and Alloplastic Material: An *In-vitro* Comparative Study on the Human Osteoblast Cell Line

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Abstract

Objectives: To investigate the effect of different bone graft substitutes on osteoconduction and mineralization in bone cells derived from the osteoblast cell line hFOB 1.19.

Methods: Osteoblast cells were cultured and placed on different bone graft materials, including Bio-Oss (xenograft), M bone (alloplast), Osteon II (alloplast), HXT1, and HXT2 (hybrid between xenograft and TCP). The concentration of elements in bone grafts was analyzed by X-ray fluorescence (XRF). The vitality test was evaluated by the methyl thiazolyl tetrazolium assay (MTT) after 1, 3, and 7 days. Alkaline phosphatase (ALP) activity was measured at 3, 7, and 14 days. Alizarin red S staining assay was performed at 7, 14, 21, and 28 days. The data were analyzed using ANOVA along with Tukey's honestly significant difference test.

Results: The cell viability rate was significantly higher in Osteon II and HXT2 compared to the other materials ($p<0.001$). On day 14, Osteon II and the HXT2 group had higher levels of ALP activity than the Bio-Oss group ($p<0.05$). Alizarin red assay showed that Osteon II had the highest mineralization ($p<0.001$) at days 14, 21, and 28, followed by HXT2 and Bio-Oss respectively.

Conclusions: Osteon II, an alloplastic bone graft, and HXT2, a newly developed hybrid between xenograft and TCP, exhibited high viability rates and expression levels in mineralized tissue cells of the osteoblast cell line hFOB 1.19 *in vitro*.

Keywords: bone graft, bone tissue engineering, hybrid bone graft, osteoblast cells, xenograft

Introduction

Bone volume lost following tooth extraction is a problem for dental implant treatment. About two-thirds of the bone loss may occur within the initial three months post-extraction.[1] Several procedures have been developed to engineer lost bone, including guided bone regeneration (GBR), which involves filling bone defects with graft material to reconstruct alveolar bone and minimize future loss.[2]

Autograft is the standardized choice for a bone graft since it can possess all three requirements, which are osteogenesis, osteoinduction, and osteoconduction, for bone regeneration.[3] However, the autogenous bone has numerous benefits, its limitation is the quantity to collect for large defect and the fastest resorption of all bone grafting materials.

Xenograft is bone tissue collected from various species of the host. The use of xenograft is a material of choice for dental implant treatment. Deproteinized and defatted bone (Kiel bone or Oswestry bone) has a lower immunological response, but it loses its osteoinductive properties.[4] Bio-Oss® (Geistlich, Wolhusen, Switzerland) is the leading commercial deproteinized bovine bone substitute for regenerative bone dentistry worldwide. Bio-Oss® can be integrated and then replaced by new bone formation in dogs after implantation for 4 months. It fulfills the criteria of an osteoconductive material.[5]

Allograft is tissue collected from one individual and grafted into another individual of the same species. Allografts do not provide osteogenic characteristics without live cells. The extent of osteoinductive and osteoconductive properties, and the methods of graft processing, affect the mechanical strength of allograft. Despite the benefits of allografts, there have been reports of HIV and hepatitis C virus (HCV) infections, as well as the risk of bacterial microbiome contamination and unknown viral infections.[6,7]

Alloplastic bone grafts are designed to replicate the natural properties of bones. Common synthetic materials used in orthopedic and dental treatments include metals (e.g. nickel-titanium), calcium phosphate ceramics (e.g. hydroxyapatite and tricalcium phosphate), and decellularized bone matrix, polymers (e.g. polymethylmethacrylate).[8] Biphasic calcium phosphate (BCP) composed material of beta-tricalcium phosphate (β-TCP) and hydroxyapatite (HA). Altering the composition (HA/β-TCP ratio) and/or crystallinity of BCP bioceramics can affect their bioreactivity. BCP bioceramics are currently approved for usage in orthopedic and dental applications as a substitute or supplement with autogenous bone.[9] The HA/TCP ratio appeared to be inversely linked to the quantity of bone formation and filler material degradation. A higher ratio of HA results in a lower resorption rate. Consequently, the resorption rates of autogenous bone and BCP 20/80 were high, while those of BCP 80/20 and BCP 60/40 were low.[10]

Hybrid bone graft is a combination of different types of bone grafts, with aims to enhance and improve specific properties derived from each type of bone graft. At present, there is still a limited prevalence of hybrid bone grafts, and there is a scarcity of studies in this area.[11]

The activity of alkaline phosphatase was especially important as it played a critical role in initiating the mineralization process of bone development.[12] To determine the degree of mineralization in cell cultures, the specimens underwent staining with Alizarin red S (ARS), which is a well-established technique used to assess the presence of calcium-rich deposits within cultured cells for decades.[13,14]

The objective was to assess and contrast the osteoconductivity and mineralization of alloplastic and hybrid bone graft materials (Xenograft sintering and coating with-β TCP) in comparison with a commercially available xenograft material, specifically investigating the mineralization process using a human fetal osteoblast cell line (hFOB1.19) model.

Materials and Methods

Materials

Bio-Oss® (Geistlich Pharma, Wolhusen, Switzerland) was a xenograft material used as a positive control group. M-bone (National Science and Technology Development Agency: NSTDA, Pathum Thani, Thailand) and Osteon II (GENOSS Co., Suwon, Korea) were commercially alloplastic bone graft materials with a ratio of HA/β-TCP of 30/70. Prototype hybrid xenograft TCP 1000 (HXT1) (OSS HYDROXY COMPANY LIMITED, Nonthaburi, Thailand) and prototype hybrid xenograft TCP 1200 (HXT2) (OSS HYDROXY COMPANY LIMITED, Nonthaburi, Thailand) were hybrid materials, in which xenografts (bovine bone) were coated with

β-TCP at sintering temperatures of 1000°C and 1200°C, respectively.

Osteoblast cells hFOB 1.19 were obtained from ATCC (American Type Culture Collection, Manassas, VA). The complete media for this cell line consisted of a 1:1 combination of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium with 2.5 mM L-glutamine (without phenol red). The following ingredients were added to the base medium to create the complete growth medium: 0.3 mg/ml G418; fetal bovine serum to a final concentration of 10%. Cultures were incubated at 37°C with 95% air and 5% CO_2 in T-75 flasks, and the culture medium was changed every 3 days. Mineralization was induced with osteogenic media (OstM) containing 10 mM β-glycerophosphate, 50 µg/ml L-ascorbate, and 10-7 M dexamethasone. Cultures were incubated at 37°C with 95% air and 5% CO_2, and the culture medium was changed at regular intervals, specifically every three days, to ensure optimal growth conditions for the cells.[15]

The osteoblast cell line utilized in this study is hFOB 1.19, which is derived from human fetal tissue. This cell line has been established as a valuable experimental model for investigating osteoblast biology, particularly in the context of drug development and biomaterial engineering. The choice of hFOB 1.19 is advantageous due to its human origin and its ability to provide a homogeneous, rapidly proliferating system for studying osteoblast differentiation and physiology.

Methods

X-ray fluorescence (XRF)

Each sample will be mounted on carbon tape and then examined with a Micro-XRF spectrometer (M4 TORNADO PLUS) to investigate the concentration of elements present in the samples. The scan was conducted with a pixel time of 20 ms/pixel, and a total acquisition time of 9 minutes. The pixel size was set to 40 µm, and the X-ray tube operated at 50 kV. The detection limit of the Bruker M4 TORNADO PLUS micro-XRF system typically ranges from 1 to 100 ppm (parts per million).

In vitro biological studies

Cell viability by MTT assay

Cell viability was evaluated using the MTT assay, which involves the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by living cells.

To ensure accuracy, all experiments were performed in triplicate. Eight sequential trials were conducted to optimize cell density and graft quantity. Initially, 10,000 cells/well in 96-well plates allowed clear formazan visualization (Experiment 1). For 24-well plates, 20,000 cells/well was optimal by day 7 (Experiments 2-3). However, 100 mg of graft caused significant cell death (Experiment 4). Due to limited graft availability, M-bone from Chiang Mai University was used (Experiment 5), and 50 mg was found suitable in 24-well plates (Experiment 6). Pre-soaking grafts for 24 h improved early viability (Experiment 7), but was excluded for being unrepresentative. The final condition used 7.5×10^4 cells/well with 50 mg of non–pre-soaked graft (Experiment 8).

The hFOB1.19 cells were seeded at a density of 7.5×10^4 cells per well into a 24-well plate (Greiner bio-one, Germany). Cells were treated with 50 mg of various bone grafts as described above for 1, 3, and 7 days, and the osteogenic medium was changed every 3 days. Cultures were incubated at 37°C with 95% air and 5% CO_2. Each cell-containing well was added with 250 µl MTT solution (5 mg/ml MTT dissolved in PBS: Phosphate buffer saline) and 250 µl DMEM F-12. The plate was incubated in a CO_2 incubator at 37°C for 3 hours. The medium was then removed, and formazan crystals were dissolved in 1,000 µl of DMSO and absolute ethanol (ratio 1:1). After incubation for 10 minutes, absorbance was read in a 96-well plate at a wavelength of 540 nm with a reference wavelength of 690 nm. The data were recorded using a microplate reader (TECAN Sunrise™, Austria). Equation (1) was used to calculate cell viability percentage. No material is the group that is 100% comparable in each experimental group and each day in MTT result.

$$\text{Cell viability} = \frac{OD_s}{OD_n} \times 100 \quad (1)$$

where ODs = sample optical density and ODn = No bone graft sample optical density

Bio-Oss was selected as the positive control group in this study due to its widespread use, established reputation, and strong support from numerous previous research studies.

Alkaline phosphatase activity

Each bone graft material (50 mg) was added to 24-

well plate (Greiner bio-one, Germany). Approximately 7.5×10^4 cells of hFOB1.19 were added to each well with bone grafts for 3, 7, and 14 days with 2 ml of osteogenic media. The osteogenic media were collected on days 3, 7, and 14 of the experiments to measure ALP activity using an ALP detection kit and fluorescence (Sigma-Aldrich, USA). Briefly, 20 µl of each sample was added to a 96-well plate (duplicates from each well) and incubated at 65°C for 30 minutes, then cooled down to room temperature with ice. Twenty µl of dilution buffer, 160 µl of fluorescent assay buffer, and 4 µl of the diluted substrate were added to each well. The plate was mixed and incubated in a dark room at room temperature for 15 minutes. Finally, the fluorescence was read by a Spark multimode microplate reader (Tecan, Switzerland) set to 360 nm excitation and 440 nm emission.

Alizarin red-s staining: microscopy and assay

The hFOB1.19 cells were seeded at a density of 7.5×10^4 cells per well in a 24-well plate (Greiner bio-one, Germany). Cells were treated with 50 mg of various bone grafts, as described above, for 7, 14, 21, and 28 days, and the osteogenic medium was changed every 3 days. Nodule formation was observed on days 7, 14, 21, and 28 of the experiments by Alizarin red-S staining. Briefly, the medium from the wells was aspirated, and the cells were rinsed twice with 500 µl of PBS. The cell cultures were fixed for 15 minutes with a 4% paraformaldehyde solution in PBS. The solution was removed, and the cells were rinsed three times with PBS. The cells were subjected to staining with a 40 mM Alizarin Red S solution in deionized water, adjusted to a pH of 4.2. This process was carried out under controlled conditions: at room temperature, in darkness, and for a period of 45 minutes. After staining, the Alizarin Red S solution was aspirated, and the cells were rinsed ten times with deionized water. The removal of water was done by aspiration, and the cells were incubated in PBS for 15 minutes at room temperature. The PBS was aspirated from the wells, and the cells were gently washed with a fresh solution of PBS. After that, they underwent four rinses with deionized water and were observed using an inverted light microscope (Olympus BX41, Japan).[13,15]

The cells were destained for 15 minutes with 10% (w/v) cetylpyrindinium chloride (CPC) in 10 mM sodium phosphate (pH 7.0). The collected stain medium was added to a 96-well plate, and A SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp.) was used to measure the absorbance at 562 nm.[13,16]

Statistical analysis

Normality tests were initially conducted on the datasets. The normally distributed data were summarized as mean ± standard deviation. Statistical analysis involved a two-way ANOVA to evaluate the effects of the variables and their interactions. Tukey's test was applied post-hoc to compare the means of different groups. The analysis was performed using SPSS version 25 software (IBM, USA), with a significance level of $p<0.05$ used to determine statistically significant differences.

Results

X-ray fluorescence (XRF)

XRF analysis was conducted to study the chemical composition of the materials. Bone morphologies of each bone graft were depicted in Figure 1A. The individual element content is listed in Table 1 and was also graphically presented in Figure 1B. Elemental mapping and spectrum analysis was show in Figure 2. The XRF analysis indicated that each bone graft had a similar ratio of oxygen, calcium, and phosphorus. Calcium concentrations follow the order: Osteon II > HXT2 > Bio-Oss > HXT1 > M-bone.

In vitro biological studies

Cell viability by MTT assay

Cell viability of hFOB 1.19 cells on bone graft materials, measured by the MTT assay, was presented in Figure 3. There were significant differences between the cell-only group and the tested bone graft materials ($p<0.001$). Each tested material exhibited a significantly decreased percentage of viability compared to the no-bone-added group on the first and third days. Osteon II and HXT2 had significantly higher levels of MTT than the other tested materials ($p<0.001$). On day 7, Osteon II had the highest level of MTT (104%) among the tested materials ($p<0.001$), followed by HXT2, Bio-Oss, HXT1, and M-bone group with the lowest level (22%).

Alkaline phosphatase activity

Alkaline Phosphatase (ALP) was involved in osteogenic differentiation, playing a significant role in initiating the mineralization process and forming the Extracellular Matrix (ECM). The study examined the effect of different bone graft materials on ALP release in hFOB1.19 cells by

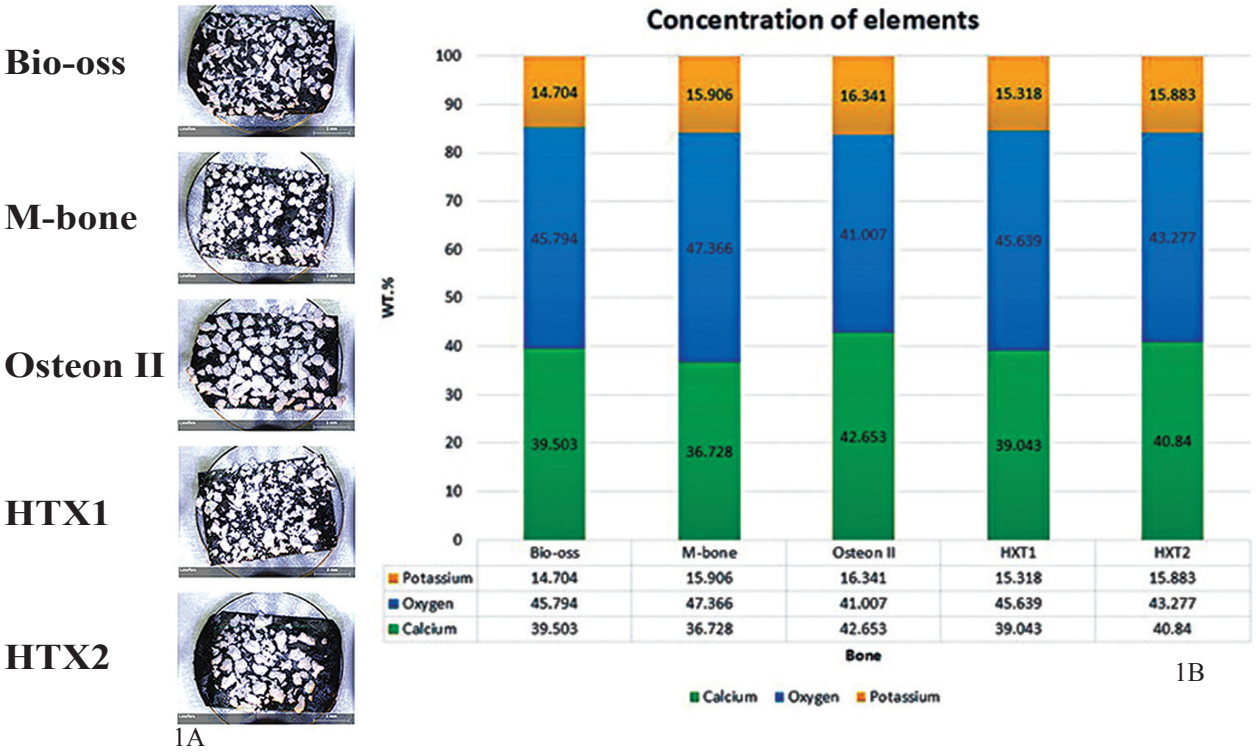

Figure 1: Bone morphologies of each bone graft are depicted in Figure 1A. The individual element content is presented in Figure 1B with a similar ratio of oxygen, calcium, and phosphorus. Calcium concentrations follow the order: Osteon II > HXT2 > Bio-Oss > HXT1 > M-bone.

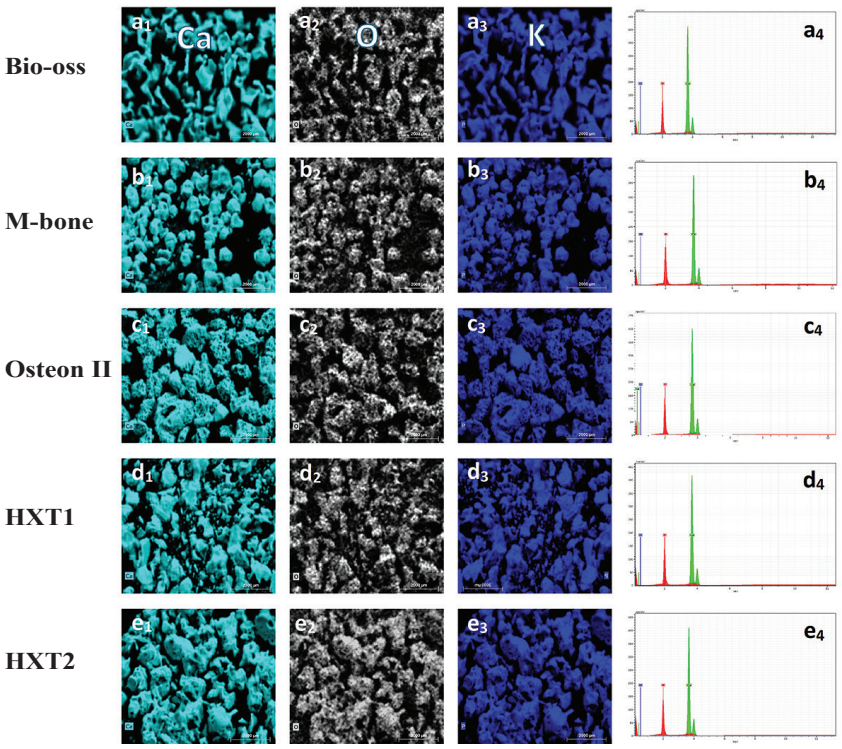

Figure 2: Elemental mapping analysis showed Ca element (a1-e1), O element (a2-e2), K element (a3-e3), The intensity of elements contents in the corresponding samples are shown in graphs a4, b4, c4, d4, e4 respectively.

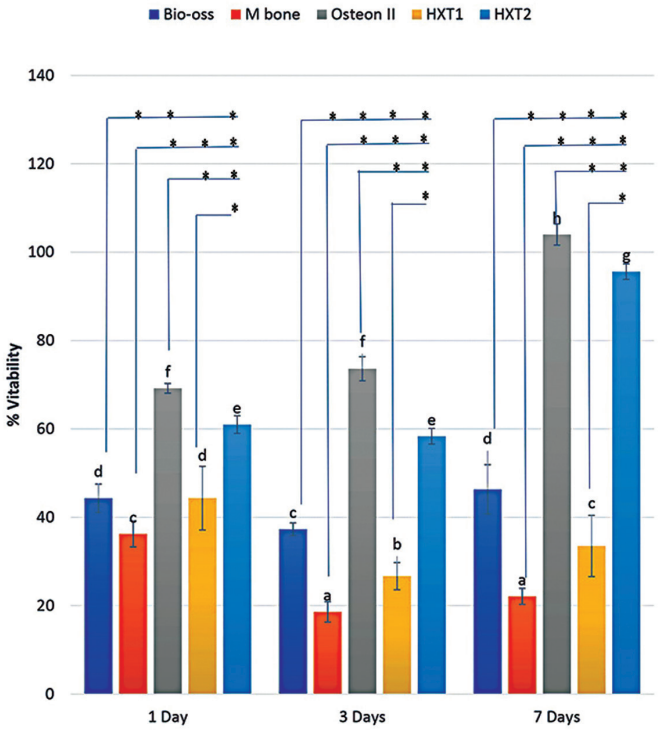

Figure 3: Cell viability measured by MTT assay result after 1, 3, and 7 days of culture on different bone substitutes. Data are reported as mean of % viability ± SD (n=15 per group). With the same letter indicating that there is no significant difference between the groups (p<0.05), *p<0.001. Osteon II and HXT2 had higher level of MTT than control group (Bio-Oss) at significantly 95%.

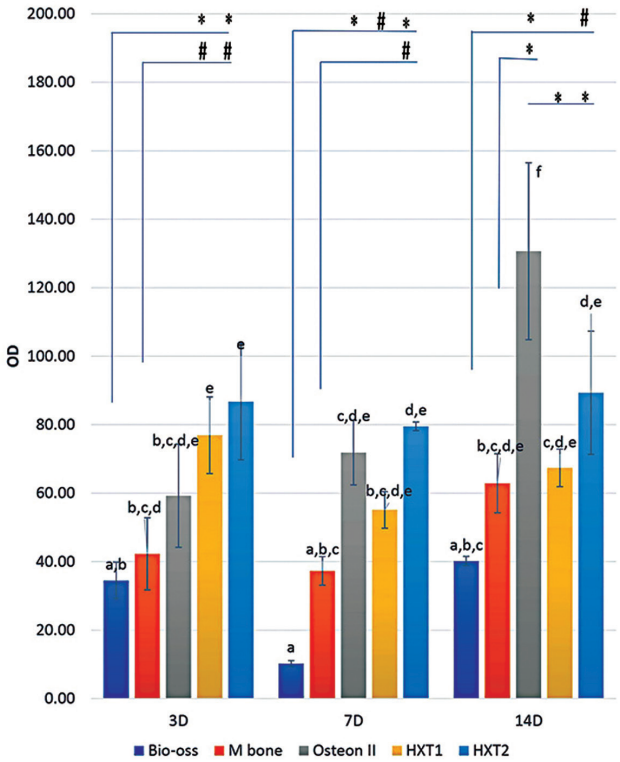

Figure 4: Quantification of ALP activities in response to each group at day 3, 7, and 14 shown in units/mg protein (Data are expressed as Mean ± SD, n=4). With the same letter indicating that there is no significant difference between the groups (p<0.05) and * p<0.001.

measuring ALP activity in the culture media.

It was found that ALP activity in every material, except for Osteon II, was slightly lower on day 7, with the highest level observed on day 14 (Figure 4). Each day of the treated group showed no significant difference in ALP activity, except for Osteon II, which significantly higher ALP activity than other tested materials was found on day 14 (p<0.001) (Figure 4). On day 3, Osteon II and HXT2 groups had significantly higher levels of ALP activity than Bio-Oss (p<0.001). On day 7, Osteon II, HXT1, and HXT2 groups had significantly (p<0.05) higher levels of ALP activity than the Bio-Oss group. On day 14, Osteon II and the HXT2 group had higher levels of ALP activity than the Bio-Oss group (p<0.05), with the highest level of ALP in Osteon II, followed by the HXT2 group.

Study on mineralization by alizarin red-s staining: microscopy and assay

Alizarin red is a marker for mineralization. When colored circle around the cells is found, means that the bone-forming cells (osteoblasts) are creating a mineralized structure. The different materials bone graft can found Alizarin red circle. The qualitative measurement of calcium deposition was examined by histochemical staining. On days 14, 21, and 28, cells were stained with Alizarin red-S, and nodule formation was investigated using an inverted light microscope (Figure 5). Nodule formation was observed on days 14, 21, and 28 in all conditions. However, cells cultured without adding any bone graft to osteogenic media indicated a clearly lower level of nodule formation. The results confirmed the importance of scaffolds in the process of mineralization.

The quantification of Alizarin Red S-stained particles was performed using the CPC extraction method, and the absorbance of the resulting solution was assessed at a wavelength of 562 nm. Every group showed increasing in the results day by day (Figure 6). On day 7, HXT1 showed significantly higher mineralization over other materials (p<0.001) (Figure 6). Osteon II showed significantly higher mineralization (p<0.001) on days 14, 21, and 28, while HXT2 and Bio-Oss were in between. M-bone had significantly lower mineralization than other tested groups on days 21 and 28. On day 28, the Osteon II group had the highest mineralization (p<0.001), and HXT2, Bio-Oss, HXT1, and M-bone decreased in order (p<0.001).

Discussion

For bone regenerating operations, xenograft bone substitutes have been used extensively. Bio-Oss® (Geistlich, Wolhusen, Switzerland) is the leading commercial bone substitute for regenerative bone dentistry worldwide. Alloplastic bone substitutes are employed due to their synthetic nature, comprising vital chemical elements found in natural bone, such as calcium and phosphate, recognized for their ability to stimulate bone regeneration.[17] Alloplastic bone substitutes offer advantages, including product quality and a reduced risk of infectious diseases, in contrast to allogeneic and xenogenic bone grafts.[6,7] However, the processing method employed for bone grafts can influence the outcomes in relation to osteoblast cells. The bone regeneration of hFOB 1.19 osteoblast cells on five bone graft substitutes was compared, and cell viability activity was studied by the MTT

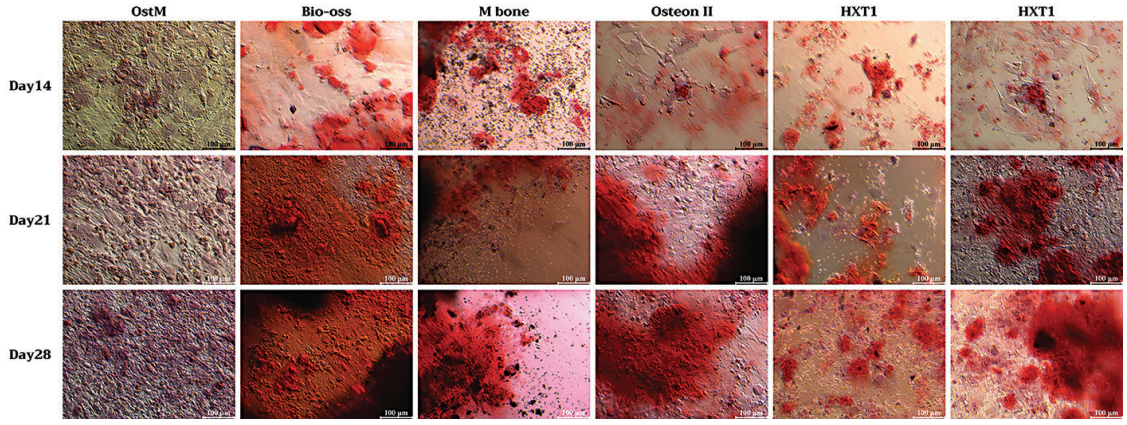

Figure 5: Alizarin red-S staining revealed the calcium nodule under the inverted light microscope.

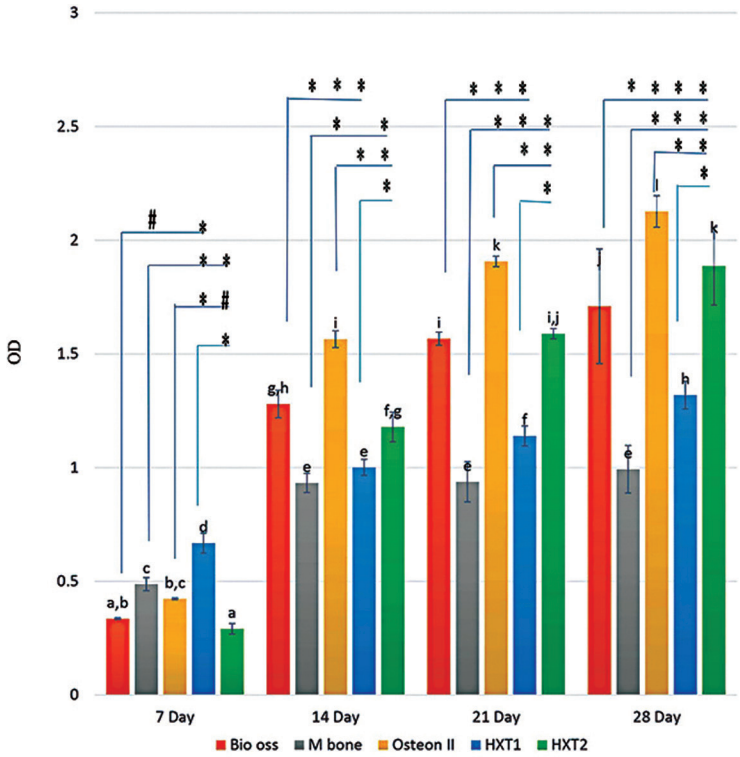

Figure 6: Alizarin Red S–stained particles were quantified by the CPC extraction method, with the absorbance of the extracted solution measured at 562 nm. (n=12, With the same letter indicating that there is no significant difference between the groups $p<0.05$, # $p<0.05$, * $p<0.001$.)

assay at 1, 3, and 7 days. The ALP activity was studied at 3, 7, and 14 days. Alizarin red staining was studied at 7, 14, 24, and 28 days.

The MTT assay findings showed a statistically significant interaction between time and group variables, influencing cell viability. The *p*-value of less than 0.001 underscored the strong statistical significance of this interaction, suggesting that both time and group factors played crucial roles in determining cell viability. The cell viability at day 7 was in the following order: Osteon II > HXT2 > Bio-Oss > HXT1 > M-bone. The difference among the Bio-Oss group at 1, 3, and 7 days was statistically significant ($p<0.001$). Nader *et al*.,[18] showed that Bio-Oss had the lowest MTT among other xenograft and alloplastic bone grafts. Kübler *et al*.,[19] reported the same result when comparing Bio-Oss with another bone graft. The results show that Osteon II and HXT2 have significantly ($p<0.001$) higher viability than the other groups in every period of the experiment. In contrast to our results, Nader *et al*, found that Bio-Oss and Osteon material had no difference in cell viability on SaOS-2.[18] Cell viability was not affected by the type of bone graft, even if the same type as HXT1 and HXT2 had a different viability rate. HXT2 exhibited a higher vitality rate compared to HXT1, indicating that the sintering process at 1200°C enhanced its compatibility more effectively than the 1000°C sintering conditions. Furthermore, HXT2 demonstrated a vitality rate surpassing that of Bio-Oss but falling short of Osteon II. Notably, HXT2 combines the properties of xenograft and biphasic calcium phosphate (BCP) materials, offering a unique mixed of characteristics that may be advantageous in specific applications. Alcaide *et al*.,[20] proceed a study involving the cultivation of SaOs-2 cells on HA/β-TCP discs, a good biocompatibility and a high percentage of viable cells on these discs. Saldana *et al*.,[21] reported that the viability of human mesenchymal cells on BCP remained stable over a four-day period. The higher performance of Osteon II compared to Bio-Oss may be attributed to the higher solubility of the β-TCP compound, potentially facilitating subsequent bone growth within the hydroxyapatite particles. Nevertheless, our findings revealed that cells cultured on M-bone, a BCP, exhibited significantly lower cell viability compared to other groups. Cell growth and proliferation are influ-

enced not only by the graft's chemical composition but besides by surface roughness and porosity. The porosity surface and pore sizes can impact cell growth and morphology.[22] Another factor could be the effect of these materials on the medium's pH, as the release of phosphate ions might affect cell growth too.[23]

Similar outcomes were reported by Schmitt *et al*., after cultivating osteoblast-like cells from bovines onto several bone substitute materials. The authors observed the non-adding bone substitution had the highest proliferation. Bone substitute materials have been shown to support cell differentiation effectively. However, they often exhibit a reduced rate of cell proliferation. Specifically, Bio-Oss was found to have the lowest proliferation rate among the materials examined in this study.

Alkaline phosphatase is highly expressed in mineralized tissue cells and plays a crucial role in the creation of hard tissue.[24] Our study showed that Osteon II had the highest ALP activity at 14 days. Nader *et al.,* observed that Osteon material, followed by Bio-Oss, had the higher ALP activity.[18]

Even though we measured each type of bone graft equally, they have different densities. Miron, Richard *et al*.,[25] demonstrate how bone graft seeding density has a significant impact on *in vitro* testing of bone-grafting materials. Osteoblast adhesion and cell proliferation were lower when cells were seeded on high-density bone graft when compared to low-density bone graft. In contrast, alkaline phosphatase (ALP) and alizarin red staining were significantly increased on high-density bone graft. Bone graft materials can influence cell differentiation and bone formation based on their chemical characteristics and how they affect the concentration of Calcium ions. The absence of calcium deficiency in BCP's composition, coupled with its lower textural properties, restricted excessive calcium ion uptake. This balanced calcium environment allowed osteoblasts to develop a functional phenotype and exhibit enhanced alkaline phosphatase activity.[21] XRF showed that the concentration of calcium followed this order: Osteon II > HXT2 > Bio-Oss > HXT1 > M-bone, and this result is the same order as cell viability, ALP assay at 7 days, and ARS assay at 21 and 28 days. Even if Osteon II has the highest Calcium element, at 7 days of ARS assay, they didn't have the highest level of Calcium deposition. This result showed that osteoblast cells differently deposited calcium elements in each bone graft.

Calcium deposition was significantly higher in Osteon II compared to other groups, even if M-bone was the same alloplastic bone graft material that had a ratio of HA/β-TCP is 30/70. ARS assay shows Osteon II had the highest calcium deposited at 14, 21, and 28 days. HXT 2 had the second one, followed by Bio-Oss. The top three highest are different types of bones.

In a rabbit study, small bone particles size 0.25-1 mm were found to be more effective in promoting osteogenesis than larger bone graft materials measuring 1.0-2.0 mm.[26,27] In contrast, a study conducted in dogs reported that β-tricalcium phosphate (β-TCP) particles sized 1-2 mm, in combination with recombinant human platelet-derived growth factor-BB, resulted in significantly greater new bone and cementum formation than smaller particles (0.25-1 mm)[28] Similarly, in augmentation procedures utilizing various grafting materials including anorganic bovine bone, allografts, biphasic β-TCP with hydroxyapatite (alloplasts), and demineralized bovine bone mineral grafts with larger particle sizes (1-2 mm) yielded superior outcomes compared to those with smaller particle sizes (0.25-1 mm)[29-31] Furthermore, Riachi *et al*.,[32] In the investigation of sinus augmentation procedures for implant placement, it was found that the average particle size of Bio-Oss (1 mm) was considerably smaller than that of Cerabone (2.7 mm). Radiographic analysis revealed that Bio-Oss underwent significantly greater volumetric reduction over time compared to Cerabone.

The particle size ranges of the bone graft materials used in this study were as follows: Bio-Oss® (0.25-1 mm), M-Bone (0.5-1 mm), Osteon II (0.2-2.0 mm), HXT1 (0.2-1.5 mm), and HXT2 (0.5-2 mm). The results indicated that larger bone particles facilitated superior bone regeneration compared to smaller particles, which aligns with reported findings.

Including implant surface roughness data would significantly improve the completeness of this study, as surface topography critically affects osteoblast response and osseointegration. Previous research shows that moderate roughness (Ra around 1-2 μm) enhances cell adhesion, proliferation, and differentiation better than either very smooth or very rough surfaces.[33,34] Specifically, Le Guehennec *et al*.,[33] highlighted that rough surfaces improve protein adsorption and integrin-mediated cell attachment, which are essential for bone formation. One review noted that roughened titanium sur-

faces achieved higher osteogenic marker expression and improved bone–implant contact in both *in vitro* and *in vivo* settings.[35]

Our observations, showing superior mineralization in HXT2 and Osteon II groups, align with known effects of material processing and composition. Studies on biphasic calcium phosphate (BCP) ceramics reveal that sintering temperature significantly alters microstructure and roughness. For instance, increasing the sintering temperature from 1000°C to 1300°C leads to grain growth and changes in porosity, which directly correlate with mechanical strength—an indirect indicator of surface topology.[36] Additionally, SEM analysis of HA/β-TCP mixtures confirms that β-TCP-rich surfaces exhibit more pronounced roughness and porosity than HA-rich ones.[37] Given that HXT2 is sintered at a higher temperature and Osteon II has a biphasic structure, their favorable performance may stem from these roughened surfaces.

However, it's important to note that this *in vitro* study exclusively demonstrates the effects of different bone graft substitutes on osteoconduction and mineralization in osteoblast cell line, which differs from the conditions in the human living body. Furthermore, the decision-making process for selecting the appropriate bone material for bone grafting procedure requires comprehensive clinical studies. Moreover, the novel hybrid bone substitute material (HXT2) still lacks an *in vivo* study to validate its tissue biocompatibility and regenerative potential. Hence, the further clinical research is required to compared bone substitute material in terms of clinical outcomes, stability, and bone regenerative properties of the grafts.

Conclusions

Distinct bone graft materials have different impacts on the cell viability and mineralization of hFOB 1.19 osteoblasts *in vitro*. The results suggest that higher concentrations of calcium elements affect mineralization. Considering the present results, the osteoblast cell line hFOB 1.19 in the presence of the tested scaffolds demonstrated that alloplastic material (Osteon II) and newly hybrid xenograft with β-TCP (HXT2) significantly promote mineralization in osteoblast cells *in vitro*.

Acknowledgments

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Declarations

Ethics approval and consent to participate

This research has been approved by Chiang Mai University Institutional Biosafety Committee (CMUIBC A-0566006) as following guidelines for safety in the production and possession of pathogens and animal toxins used in research studies and correspond to the level of risk of disease and danger.

Conflicts of Interest

The authors of this study affirm that they have no competing interests or financial ties that could potentially influence the results or interpretation of the findings

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