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An Innovative Bioceramic Bone Graft with Platelet-Rich Plasma for Rapid Bone Healing and Regeneration in a Rabbit Model

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Abstract: This study aimed to investigate the effect of combining an innovative bioceramic α-calcium sulfate hemihydrate (α-CSH, $CaSO_4 \cdot 0.5H_2O$) bone graft and platelet-rich plasma (PRP) to accelerate bone healing and regeneration in a rabbit model. The bone graft material was implanted bilaterally on rabbit's artificially maxillary sinus defects: the right maxillary sinus received α-CSH, while α-CSH combine with PRP (α-CSH/PRP) was grafted in left site. The quantity and quality of bone formation after implantation were analyzed radiographically and histologically at 1, 2, and 3 weeks. The micro-computed tomographic results indicated that the bone density of sinus implanted with α-CSH increased and defect volume decreased most after 2 weeks. In histological analysis, both hematoxylin and eosin and Masson trichrome staining of α-CSH/PRP displays better bone healing and regeneration progress than α-CSH after 2 weeks implantation. Therefore, the innovative α-CSH combined with PRP was revealed to be useful in accelerating bone healing and regeneration for the successful defect treatment.

Keywords: α-calcium sulfate hemihydrate; bioceramic bone graft; platelet-rich plasma; bone healing; bone regeneration

1. Introduction

Bone loss treatment due to trauma, infection, tumor, tooth extraction, and various other diseases is a challenge for orthopedic and oral maxillofacial surgeons [1–4]. In the

oral cavity, the challenge is becoming more severe in treating large bone loss, especially in the posterior maxilla region, due to the lower bone density than other sites, which affects the dental implant installation for rehabilitation purposes [5–9]. Some studies reported the technique with aim to increase primary implant stability and the initial bone-to-implant contact percentage in a poor bone density at maxillary region such as undersized implant preparation, osteotomy technique to condense bone around the implant, osteodistraction osteogenesis, and the ridge splitting technique. However, those techniques seem to show an ineffectiveness to improve implant stability and bone density, and also exhibit various complications post-surgery [10].

The use of autogenous bone graft that does not cause a host response is often a solution in facilitating healing and regeneration of bone defects, even it is still the gold standard of all types of bone graft [1,4,6,11]. However, the various problems that arise when harvesting bone grafts from the donor site have resulted in the emergence of several bone grafts, which have been suggested as alternatives that can replace autogenous bone grafts in treating bone defects [1,4,6,11].

All bone graft materials encourage osseointegration improving bone quality and quantity, as well as the process in varying times depending on the type and characteristics of the material. The ideal bone graft will show a remodeling process that is identical to the bone [12]. Bone healing and regeneration is a long-term process requiring a total of about 3 to 6 months [4,13]. Meanwhile, delayed healing will cause new problems such as increased infection, prolonged treatment, and implant instability [5]. Therefore, tissue engineering, which combines a biocompatible scaffold, osteogenic cells, and growth factors seems to be promising in treating bone defects [2,14,15].

Bone tissue engineering involves the collaboration of various professions such as scientists, engineers, and surgeons to create a good bone graft to increase bone repair and regeneration. Since 1980, numerous studies and review papers on bone tissue engineering have been published and are growing from year to year, indicating that the issue of bone tissue engineering is rising in demand [16,17]. In the dentistry field, bone tissue engineering is also increasing rapidly, especially bone graft application as a scaffold [18]. The bone graft as a scaffold mostly concerns using a fixation device such as a screw and plate to stabilize the scaffold in the defect area [19–22], which potentially leads to fracture of both the host and the scaffold [23,24]. This issue serves as an evidence-based approach for the profession involved in selecting techniques and finding other alternatives treatments of bone defects by still using bone graft as a scaffold [25].

Bone grafts used as a scaffold in tissue engineering should be osteoconductive, osteoinductive, and biocompatible [15,26–29]. Based on our previous study, an innovative α-calcium sulfate hemihydrate (α-CSH, $CaSO_4 \cdot 0.5H_2O$) bioceramic bone graft has met the criteria as a scaffold that is potentially used in tissue engineering with its osteoconductive properties derived from a bone graft framework that allows bone and blood tissue to grow on its surface [12]. The α-CSH osteoinductive is associated with absorption of calcium, which induces osteogenic activity and increases local acidity, thereby promoting growth factor release in the defect area, while the biocompatibility of α-CSH is related to non-irritating, non-toxic, and is not at risk of immunological rejection [12,24,30,31]. However, the α-CSH properties need to be improved to accelerate better bone healing and regeneration. Platelet-rich plasma (PRP), which is a platelet concentrate containing various autologous growth factors, is often used to promote bone regeneration, although its use still raises various controversies related to the effectiveness of platelet concentrates in wound healing and tissue regeneration [2,6,9,13,32–36]. Nevertheless, numerous authors stated the potential of autologous platelet concentrate use in several orthopedics and oral surgery scenarios, which not only stimulate bone regeneration but also acted as local hemostatic, facilitate wound-healing, and can be used as medication-related osteonecrosis to reduce pain and increase the quality of life patient [37–40]. Hence, the present investigation aimed to further evaluate the potential rapid bone healing and regeneration of α-CSH bone graft combined with PRP in rabbit's artificial maxillary sinus defect for biomedical applications.

2. Materials and Methods

2.1. Materials

The material used in the current study was synthesized from calcium sulfate dihydrate (Acros Organics, Morris Plains, NJ, USA) through the microwave-irradiation method that produced α-CSH with guaranteed purity, which during the synthesis process did not involve various chemicals as described in our earlier studies [12,24,31]. The investigated sample used α-CSH combined with autologous platelet gel that is obtained from the platelet-rich plasma (PRP) of New Zealand White (NZW) rabbits. For easy classification, the α-CSH combined with PRP gel is denoted as α-CSH/PRP. While the α-CSH alone was adopted as a control group for comparison in this study.

2.2. Animal Study

The present animal study used six male NZW rabbits with average weight of 3.58 kg and aged 5 months purchased from the Livestock Research Institute (Tainan, Taiwan). All NZW rabbits received an equivalent implantation procedure and evaluation, then were sacrificed at different time points (1 week, 2 weeks, and 3 weeks) to observed the healing process with 2 rabbits involved per each time point.

2.2.1. PRP Preparation

All NZW rabbits received general anesthesia using a combination of Zoletil 50 (50 mg/mL) 15 mg/kg and Rompun (23.32 mg/mL) 5 mg/kg induction, administered via intramuscular injection. The preparation of PRP were performed immediately before surgery and approximately 20 mL whole blood was withdrawn via intracardiac puncture. The sodium citrate was added in a 20 mL sterile tube with blood and then centrifuged at 1600 revolutions per minute for 15 min, leading to the separation of red blood cells, plasma with platelets, and leucocytes. Subsequently, plasma was aspirated and transferred into another sterile tube. Plasma was centrifuged again at 2000 revolutions per minute for 15 min to separate the PRP at the bottom of the platelet button. Part of the platelet-poor plasma was removed and remaining PRP was to be used in the implantation procedure.

2.2.2. Implantation Protocol

Each NZW rabbit was assigned bilateral maxillary sinuses under sterile surgical condition (Figure 1). The skin at nasal dorsum area was shaved (Figure 1a), disinfected, and local anesthesia (0.5 ml of lidocaine with 1:100.000 epinephrine) was injected in the middle of this area (Figure 1b). A 2.5–3.0 cm vertical periosteal incision was performed at midline nasal dorsum (Figure 1c) and the periosteum was deviated laterally to disclose the nasal bone. A bilateral rectangular window was carried out by made two circular windows vertically (the distance of each window is 1 cm) using a 2.5 mm diameter trephine bur, located approximately 0.5 cm laterally to the midline nasal bone (Figure 1d), and each circular window then connected to shape rectangular window (Figure 1e). Hereafter, the investigated α-CSH/PRP (0.3 g) and α-CSH (0.3 g) samples were randomly implanted in the rectangular window. Finally, the skin and periosteum were returned and sutured to the original position.

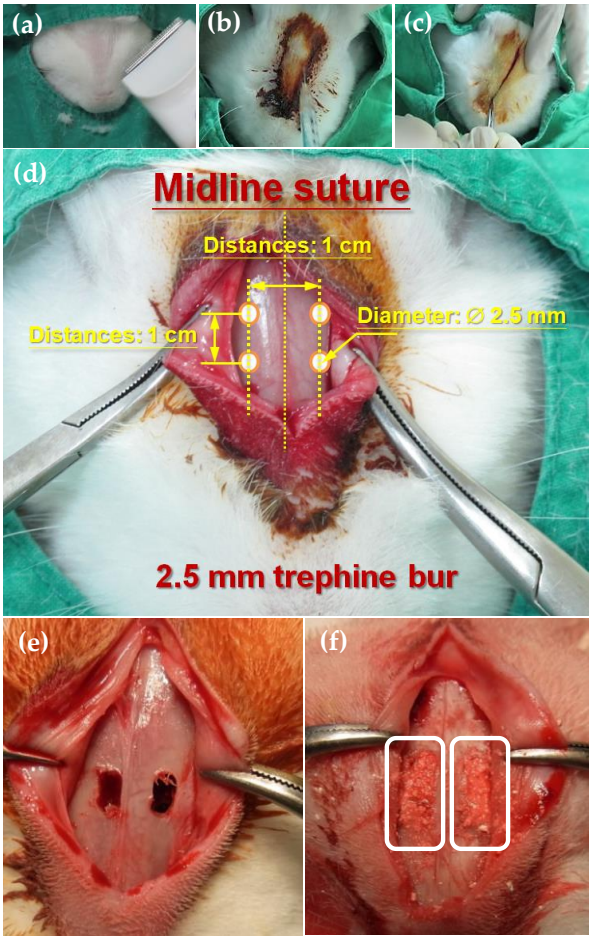

Figure 1. The implantation procedure on rabbit's created-maxillary sinus: (**a**) fur shaving at nasal dorsum area, (**b**) local anesthesia injection, (**c**) periosteal incision, (**d**) circular window, (**e**) rectangular window, and (**f**) implantation of the investigated α-CSH/PRP and α-CSH bone graft samples (randomly).

2.3. Micro-Computed Tomographic (μ-CT) Analysis

The investigated sample scanning was carried out by means of a μ-CT scanner model 1176 (Bruker Skyscan, Kontich, Belgium) under 80 kV, 300 μA tube current, 400 ms exposure time, and 18 μm of voxel resolution to scan the investigated sample. A thin copper and aluminum filter was used to diminish the noise image. The images obtained from μ-CT then reconstructed into a two-dimensional image using NRecon software (Kontich, Belgium). The regions of interest (ROI) were drawn using CTAn software v.1.18 (Kontich, Belgium), and the implanted area (8 mm diameter, 3 mm depth) was preferred as an ROI. Subsequently, the micromorphometric parameters such as bone mineral density (BMD) and new bone volume could be evaluated using the software. The regional BMD of the sample were determined from the Hounsfield unit (HU) value, which was obtained from the ROI each sample. The defect volume of the sample was obtained from the total volume of the entire ROI minus volume of the new bone.

2.4. Hystopathological Analysis

The NZW rabbits were euthanized at a prearranged timetable, their maxillary was cut, and the area involving sinuses and implant were retrieved. The samples were rinsed in sterile saline to decontamination, fixed in 10% buffered formaldehyde, and decalcified in 10% EDTA solution. The decalcified samples were washed in distilled water, dehydrated in ascending series of ethanol, embedded in paraffin wax, and cut into 5 μm sections. The sectioned samples were stained with Hematoxylin and Eosin (H&E) and Masson Trichrome,

and observed through a digital image capture pathology scanner (Aperio CS model, Leica Biosystems, Bualo Grove, IL, USA) under different magnifications.

2.5. Statistical Analysis

The microarchitectural data were analyzed using SPSS statistic software (Version 19.0., SPSS Inc., Chicago, IL, USA). The difference between multiple groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test with a value of $p < 0.05$ was indicated statistically significant.

3. Results

3.1. Bone Healing Characteristics of the Grafted Materials

Figure 2 displays the BMD in the sinuses obtained from the HU value, which was measured by μ-CT scanning. Based on the HU value obtained from the sinuses implanted by α-CSH and α-CSH/PRP, the sinuses treated with α-CSH/PRP showed a continuously increasing HU value during the observation time. Moreover, the α-CSH/PRP group experienced a significant 21% increase in HU value than the control group at the second week after implantation. The increase in HU value in the sinus group grafted with α-CSH/PRP was supported by the healing of the sinus wound over time during the observation period as shown in Figure 3.

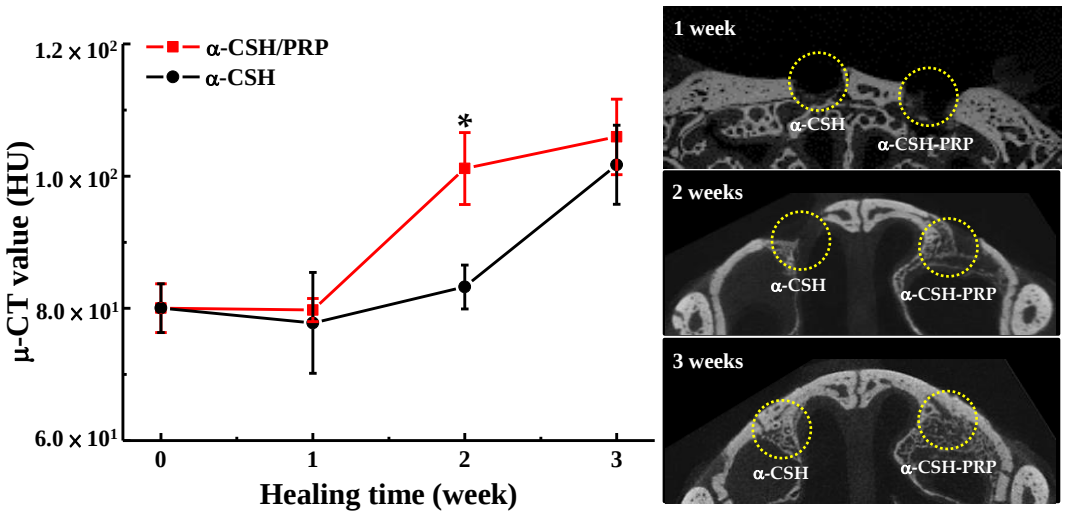

Figure 2. HU values and μ-CT images of the sinuses defect at 0, 1, 2, and 3 weeks after treatment with α-CSH and α-CSH/PRP bone graft samples. (HU was obtained by determining the intensity of gray area in the ROI of μ-CT image (yellow circular area)). Week 0 is the defect area without bone graft, which to be used as the reference value. A lower intensity of gray area in the ROI was detected in both samples at week 1. The α-CSH/PRP sample exhibited higher intensity of gray area as compared with α-CSH sample at week 2 (*$p < 0.05$). Both samples presented similar intensity of gray area at week 3. The intensity of gray area in the ROI increased with healing time increasing.

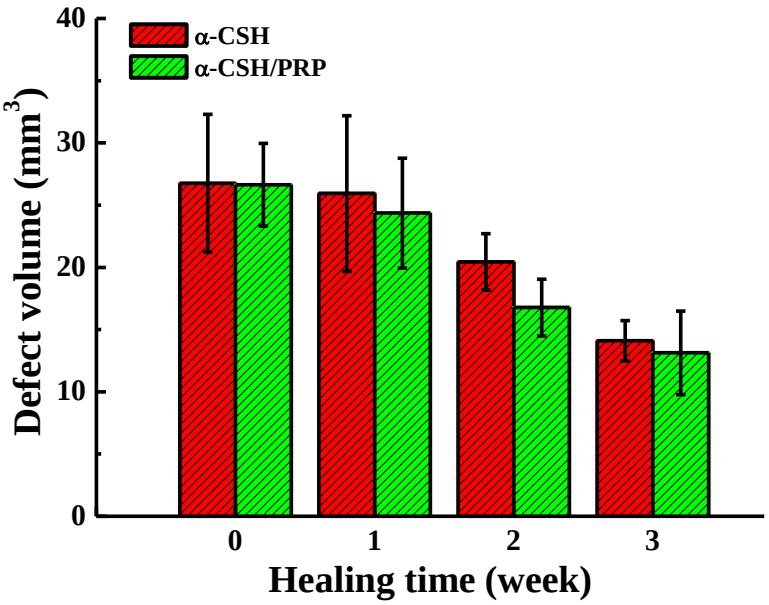

Figure 3. Defect volume of the sinuses at 0, 1, 2, and 3 weeks after treated with α-CSH and α-CSH/PRP bone graft.

Figure 3 represents wound healing in the sinuses treated with either α-CSH or α-CSH/PRP. The results express that both α-CSH and α-CSH/PRP can heal sinus wounds, yet α-CSH/PRP appear to show a faster healing progress. In line with the rise in HU value in the α-CSH/PRP group at the second week, sinuses with α-CSH/PRP experienced 37.05% total wound healing compared to sinuses implanted with α-CSH with only 23.67%.

3.2. Bone Regeneration Properties of the Grafted Materials

Figure 4 illustrates the results of H&E staining of the sinus sample treated by α-CSH and α-CSH/PRP at 1, 2, and 3-weeks evaluation periods. In the first week's evaluation, there was no significant difference in the histological appearance of the sinuses implanted with α-CSH and α-CSH / PRP. Sinus grafted with α-CSH at two-week evaluations showed granulation tissue and increased osteoblasts in the area around the bone graft, indicating bone regeneration. The area circled with a yellow dotted line demonstrates the renewal area by bone graft, while the circle with the blue dotted line reveals the bone graft placement. Sinuses treated with α-CSH/PRP at two weeks demonstrated the presence of granulation tissue, fibroblasts, and more osteoblasts surrounding grafting material indicating better bone renewal than the control group. The area circled in the green dotted line represents the implanted α-CSH/PRP and tissue renewal. Moreover, it appears that the aggregation tissue is denser compared to the control group, which suggests that the regeneration phenomenon is more pronounced. In the third week, both the α-CSH/PRP and the control group as a whole showed increased bone thickness due to resorbed bone graft and established a large amount of adipose tissue.

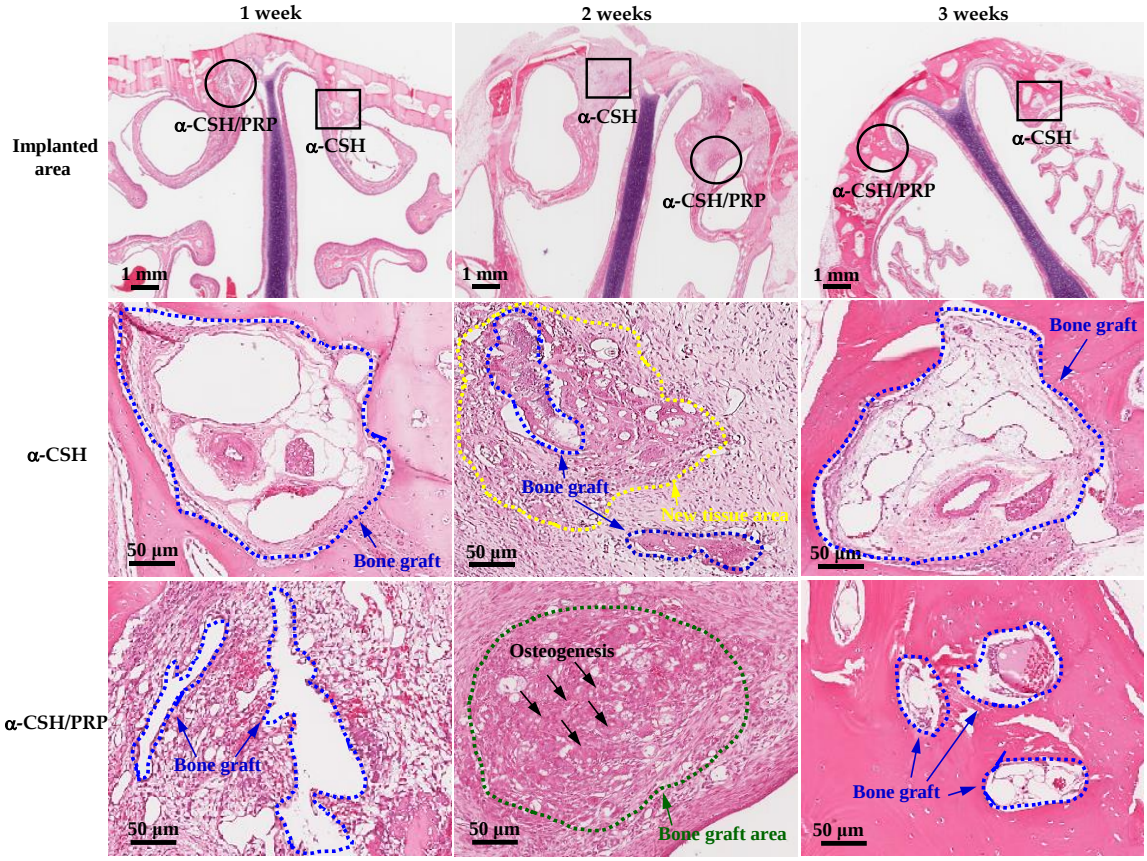

Figure 4. H&E staining results of the α-CSH and α-CSH/PRP at 1, 2, and 3 weeks after implantation. (The higher magnification images were taken from the investigated samples in each time point marked as black circular and rectangular areas, respectively).

Figure 5 was obtained from Masson trichrome stain analysis consistent with H&E staining, which overall shows that in the two-week observation period there is a significant increase in granulation tissue and fibroblasts around α-CSH/PRP. As demonstrated by H&E staining at two weeks after implantation, the control group displays granulation of tissue, indicating bone regeneration. The area circled in the control group shows repaired tissue around the α-CSH placement. There is an increase in osteoblasts, and the tissue arrangement is relatively irregular in areas, which are also part of the new tissue. Two weeks after α-CSH/PRP implantation showed granulation tissue marked with red arrows, new denser tissue around the implant (yellow dotted line), and angiogenesis (indicated by black arrows). This finding also indicated that α-CSH/PRP showed a better regeneration phenomenon than the control group.

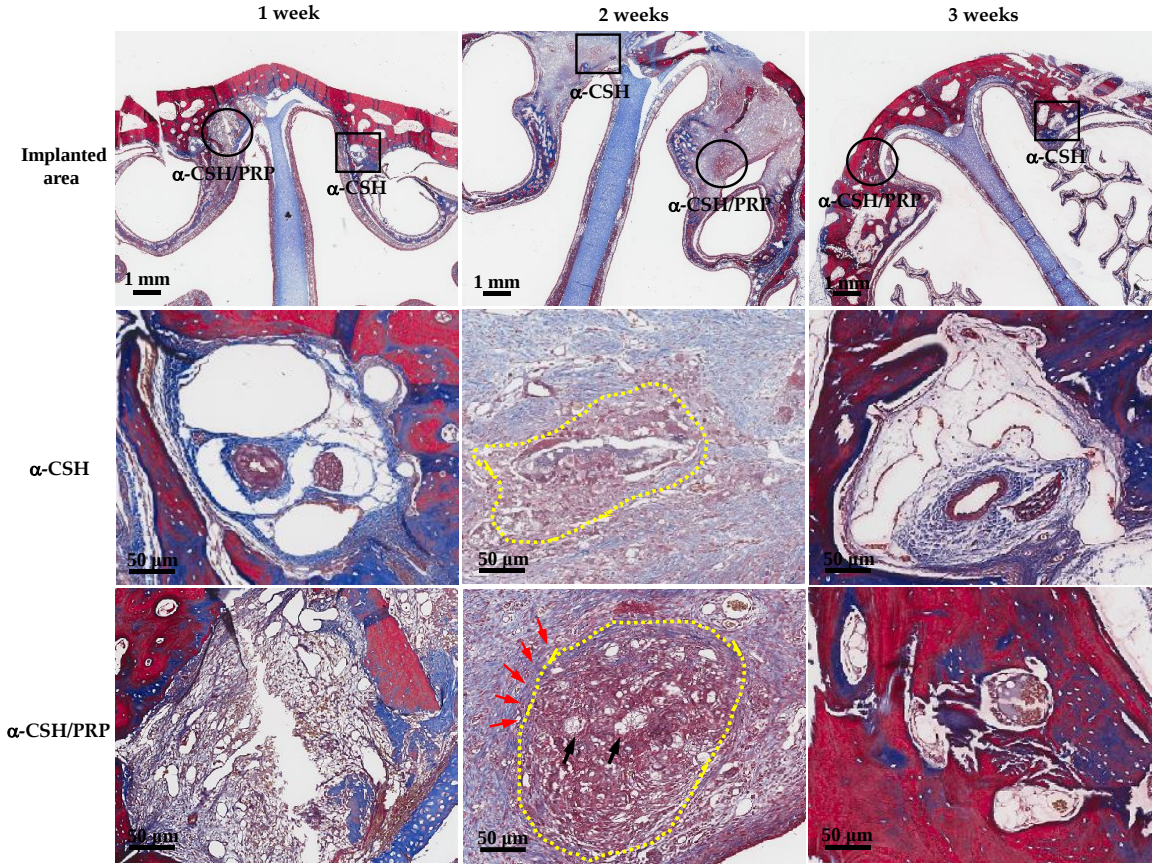

Figure 5. Masson trichrome staining results of α-CSH and α-CSH/PRP at 1, 2, and 3 weeks after implantation. (The higher magnification images were taken from the investigated samples in each time point marked as black circular and rectangular areas, respectively).

4. Discussion

The choice of bone graft material can affect the success of healing and bone regeneration during osseointegration in areas experiencing bone loss, which is estimated by BMD parameters, volume defects, and histology of the sample studied. Since BMD is relevant to the HU value [41], in present study, BMD can be accessed via HU value, which is determined from the μ-CT imaging system. In the current study, the trend of increasing HU value in α-CSH bone graft combined with PRP showed an increase in BMD in the sinus area supported by a decrease in sinus defect volume two weeks after implantation. The rise in BMD indicates an improvement in bone quality influenced by the healing period, which has an impact on bone regeneration [42,43]. Several studies have demonstrated the increased performance of PRP in bone regeneration when combined with an autogenous bone graft or mesenchymal stem cells [2,6,8,14,32,44,45]. Although autogenous bone graft is the gold standard in treating various bone defects, its use causes some specific problems such as limited stock availability, extended surgical time, and complications in the donor area such as pain, easy infection, and prolonged healing time [1,8,46,47].

The α-CSH bone graft resembles autogenous bone graft in that it promotes osseointegration with superior properties such as high purity, high calcium, quick setting, low temperature, bone booster formation, and ease to handle [12,24]. However, the α-CSH bone graft itself exhibited a slow progression in elevating BMD. A similar incident occurred in our previous study, which revealed a better progression of increasing BMD existed at 8 and 12 weeks after α-CSH implanted in the defect [12]. Nevertheless, the research using the combination of PRP with mesenchymal stem cell or an autogenous bone graft on in vivo sinus floor elevation rabbit seems to be in line with our study that increased bone volume in

the second week after implantation [8]. Another study that combined PRP and autogenous bone graft in treating rabbit's artificially calvarium defect demonstrated that new bone formation at four weeks after receiving autogenous graft with the combination of PRP was the same as a new bone formation that only obtained autogenous graft without PRP at 12 weeks post grafted [9]. The increase in bone volume and the reduction in defect volume in a short time can be provoked by the property of PRP, which increases the formation of new bone and accelerates wound healing [8]. Therefore, PRP combined with α-CSH can contribute to favorable conditions to speed up bone regeneration.

The histological evaluation of α-CSH and α-CSH/PRP indicated the appearance of bone regeneration by the second week. However, α-CSH/PRP showed better bone regeneration than α-CSH because both new tissue and osteoblasts were more common in the experimental group. Moreover, in the α-CSH/PRP group, angiogenesis was seen, which is essential in providing nutrition to the defect area to accelerate healing and lead to bone regeneration [48–50]. The use of PRP is known to regenerate bone in a short time due to the various bioactive molecules properties along with their physiological functions to induce bone regeneration in the defect area, such as adhesive protein, which services in intercellular interactions, adhesion, and osteoblast migration; growth factors, cytokines, and chemokines that function in adhesion, proliferation, migration, differentiation of osteogenic and osteoblasts, inhibit osteoclasts from occurring in bone resorption, stimulate angiogenesis, and stimulate bone repair in a fast time [9,13,34]. However, several studies have reported that PRP alone does not accelerate bone regeneration [3,6,9,35].

Using PRP alone in the treatment of defects is unfixed, caused difficulty to adapt in defect areas, and degrades very quickly [3,24]. Therefore, PRP needs to be combined with a graft material to make it more rigid. Platelet concentration and growth factor as a result of the preparation procedure are also factors that influence the effectiveness of PRP [6]. In the current study, a double centrifugation method was performed to produce optimal platelets to regenerate the bone at a concentration of 5 times higher than whole blood, while even higher concentrations could reduce osteoblast cell proliferation [6].

One of the osteoconductive properties of the graft material owns the resorption rate similar to bone formation [12]. Our previous study using α-CSH on a rabbit's artificial femur defect revealed that the graft material began to absorb, and there was an increase in bone volume at eight weeks post-implantation [12]. Another previous study also used α-CSH on rat's artificial hind leg bones defect presented the complete absorbing of graft material at seven weeks after implantation [24]. Referring to the biological process of fractured bone repairing, bone remodeling will begin with the formation of granulation tissue and soft callus, which in animal models occurs seven to nine days after injury, while in humans it occurs at two to four weeks post-trauma [51,52]. In the current study, granulation tissue is more pronounced at two weeks in defects treated with α-CSH/PRP. At the same time, angiogenesis is seen, which is a crucial part promote bone regeneration [51]. Other studies have shown that the combination of PRP and autogenous bone graft can increase the absorption of bone graft material, which is related to the new bone formation [6]. The absorption of α-CSH bone graft allow the defect to absorb bioactive component such as calcium, which responsible for re-mineralizing the defect [12,24].

The rapid regeneration of bone as a result of the combination of PRP and α-CSH provides the advantage of a single-stage dental implant clinical procedure that has the potential to fail due to the lack of osseointegration during the healing period, especially in the installation of dental implants in the maxilla region, which have a lower bone density than the mandible leading to implant instability [8,53]. Moreover, various factors that affect wound healing in the maxillary sinus such as low vascularity, low oxygen pressure, and intra-antral pressure make the use of PRP useful due to accelerating revascularization [6,33]. Several studies also have reported the use of synthetic bone grafts to be ineffective in extensive segmental defects or areas requiring load-bearing resistance due to their weak mechanical strength [54,55]. Although mechanical strength is required when restoring a defect in the jaw for chewing purposes, the use of α-CSH bone graft is successful in

treating large bone loss before implant placement, which is evident six months after implant placement, radiographs show osseointegration in the defect area, and the implant remains in a stable state [24]. As discussed above, we suggest that the combination of PRP and α-CSH can have a synergistic effect in rapid bone regeneration for clinical application. Nevertheless, the limitations of the present study are performed on small sample size, were conducted on animal samples, and have a short evaluation period. Hence, further study with a larger sample size, long-term evaluation period, and tested in clinical application is needed to support the current findings of the effectiveness α-CSH in combination with PRP.

5. Conclusions

The using of α-CSH bone graft in combination with PRP to treat maxillary sinus defect was better than α-CSH without PRP, which showed an increase of BMD, a decrease defect volume, and an increase of granulation tissue, osteoblast, and angiogenesis at two weeks after implantation. The result in the present study indicated the innovative α-CSH bioceramic with PRP is beneficial to accelerate bone healing and regeneration particularly in the case of treating large bone loss before implant placement in maxillary posterior, which requires a satisfactory healing with more osseointegration to lead to implant stability.

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