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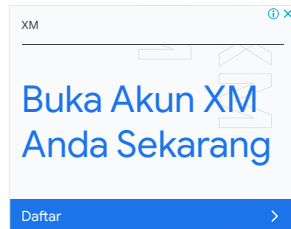
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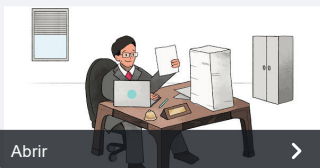
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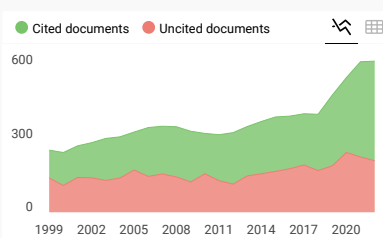
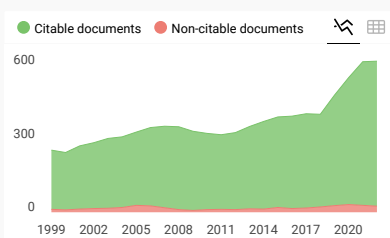
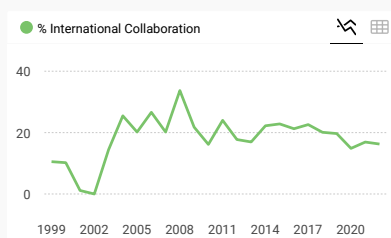
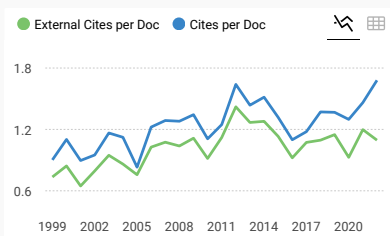
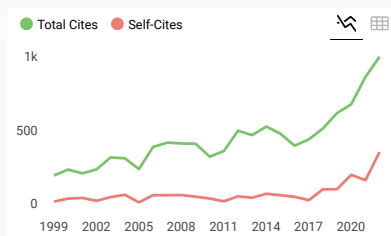
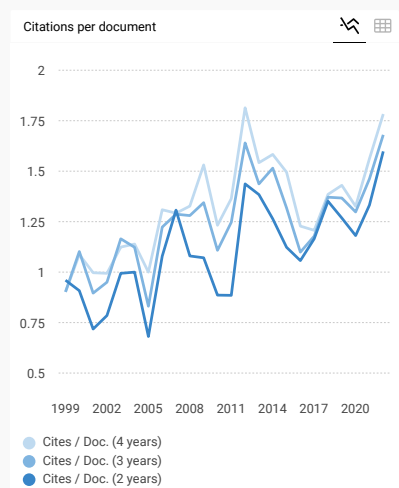
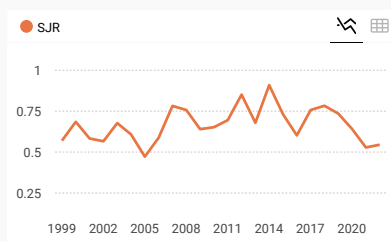


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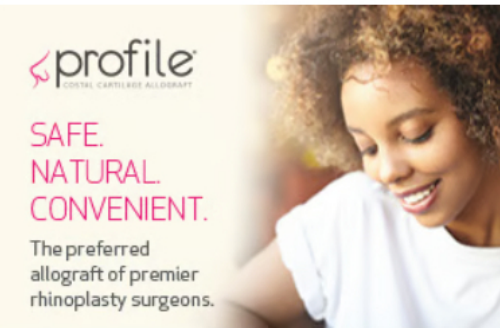
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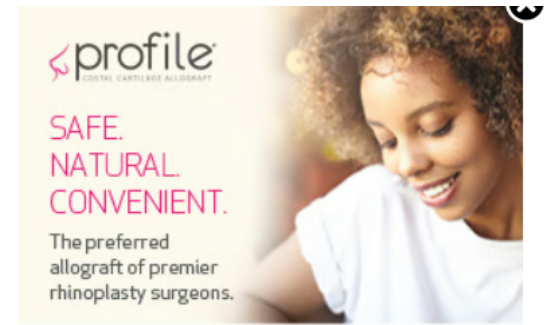
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# Alveolar Repair Using Cancellous Bone and Beta Tricalcium Phosphate Seeded With Adipose-Derived Stem Cell

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## Abstract

**Introduction:** Adipose-derived stem cells (ADSCs) have been subject of several studies due to their abundance, ease of preparation, and application in bone regeneration. We aim to compare effectiveness of alveolar reconstruction utilizing human cancellous freeze-dried graft (HCG) and beta tricalcium phosphate (BTP), both seeded with human ADSC (hADSC) and autologous bone graft (ABG).

**Material and Methods:** A 5 × 5 mm alveolar defect in 36 male Wistar rats were treated using: ABG (C), HCG–hADSC (H1), and BTP–hADSC (H2). At 1 and 8 weeks after surgery, runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osterix (OSX), and bone morphogenetic protein 2 (BMP2; g/mL) were quantified using immunohistochemistry, while bone tissue volume (BV, mm<sup>3</sup>), bone tissue volume fraction (BF, percentage), and trabecular thickness of bone (TT, mm) were assessed using micro-computed tomography (CT).

**Results:** One week after surgery, H2 was higher in RUNX2, OSX, ALP, and BMP2 than C ( $P < .05$ ). Only RUNX2 and OSX were found to be higher in H1 than C, while ALP and BMP2 were higher in H2 than H1. Micro-CT revealed that H2 had a higher TT than C and C had a higher TT than H1 ( $P < .05$ ). Eight weeks after surgery, both H2 and H1 was higher in RUNX2, OSX, ALP, and BMP2 than C ( $P < .05$ ). RUNX2 and BMP2 were found to be higher in H1 than H2. Micro-CT revealed that H2 had higher BV and TT than C and H1 ( $P < .05$ ).

**Conclusions:** Exogenous hADSC strengthened the effectiveness of HCG and BTP to accelerate osteogenesis, osteoconduction, and osteoinduction. The latter was the most successful in bone formation, followed by HCG and ABG.

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## Keywords

craniofacial, alveolar repair, tissue engineering, bone tissue engineering, stem cells, innovation

## Introduction

Since the face is the most prominent determinant of identification, changes in its form can trigger practical, physical, or psychological issues. The absence of the alveolar bone, whether complete or partial, results in a decrease in facial height.<sup>1</sup> Congenital craniofacial anomalies, such as cleft lip, alveolus, and palate, or acquired abnormalities, such as alveolar bone atrophy, craniomaxillofacial trauma, and tumors, may cause alveolar bone defects.<sup>2</sup>

Autologous bone graft (ABG) is the most common procedure for treating bone defects since it retains the properties of the initial tissue lost: osteoinduction (growth factors), osteogenesis (osteoprogenitor cells), and osteoconduction (scaffold).<sup>3</sup> ABGs include autologous bone and immunocompatible cells.<sup>4</sup> ABG's histological compatibility and nonimmunogenicity reduce the risk of immune response and infection transmission. However, there are many drawbacks to ABG, including the possibility of a donor deficiency, a small donor selection, and iatrogenic discomfort and risks involved with multistaged procedures.<sup>5,6</sup>

Other bone defects are handled with allografts from living human donors, which is a potential alternative to ABG since it comes from the same species, allografts have been recorded to have a rejection rate of over 50%.<sup>7</sup> Human cadaver as an allograft donor has the main advantage of its variability of donors, thus avoiding the need to sacrifice the structure of the donor area, and the absence of donor morbidity.<sup>8</sup> Most immunogenic agents that induce immunogenic responses may be avoided by devitalizing allografts by irradiation or freeze-drying processes.<sup>3</sup> Human cancellous freeze-dried graft (HCG) is an allograft, in which the blood and attached tissues are rinsed to prevent infectious diseases from spreading. HCG has osteoinductive and osteoconductive features, but the absence of essential bone cells makes them nonosteogenic.<sup>9,10</sup>

Tricalcium phosphate (TCP), on the other hand, is a bone regenerative substance with high biocompatibility, porosity, fast absorption, and osteoconductivity.<sup>11</sup> Beta tricalcium phosphate (BTP) is the most current edition of TCP, when compared to  $\alpha$ TCP, it degrades more quickly, giving rise to even more accelerated bone forming when exposed to bone tissue.<sup>11,12</sup> BTP has osteoconductive features but does not have osteoinductive and osteogenic features.<sup>9,10</sup>

The method of incorporating osteogenic mature cells or stem cells into three-dimensional bio-scaffolds with osteoconductive and osteoinductive features for enabling neovascularization to foster bone growth and maturation is known as bone tissue engineering (BTE).<sup>3</sup> The substrate or bio-scaffold or bio-carrier, the community of cells inside the carrier and the tissue configuration in which the incorporation phase takes place are the 3 basic components of BTE.<sup>13</sup>

Because of their ample abundance and ease of preparation, adipose-derived stem cells (ADSCs) have been the focus of many studies because of their use in bone regeneration.<sup>14-19</sup>

BTE methods utilizing human stem cells to treat bone defects in mice have been proven in several trials to have no immunological or tissue rejection side effects.<sup>20-24</sup> According to the findings, incorporating autologous bone marrow cells into ADSC guarantees allogenic skin longevity and excludes the need for immunosuppressive regimens.<sup>21</sup>

Muscle-derived stem cells (MDSC) along with  $\alpha$ TCP were not dissimilar from ABG in respect of bone volume fulfilling the 5mm<sup>2</sup> critical rat alveolar defect, according to Amaral et al.<sup>24</sup> This offers a ray of light for a further noninvasive way of repairing bone tissue defects that are as good as ABG.

The purpose of this research is to compare the effectiveness of in vivo alveolar repair in Wistar rats using a combo of HCG or BTP with human ADSCs (hADSCs). The effectiveness of these 2 BTE interventions would be compared to the conventional approach of ABG in this report.

## Methods

### Study Design

The ethical committee of veterinary medicine faculty has issued their acceptance of our protocol (IRB 2.KE.023.02.2018).

Thirty-six nonimmunosuppressed male Wistar rats, 8 to 12 weeks old and weighing 200 to 300 g, were chosen and housed in a controlled environment. A 5×5 mm critical bone defect was formed in the alveolar zone. The rats were split into 3 treatment groups in the first week and 3 treatment groups at week 8 (n=6). The defects were loaded with ABG (C); HCG-hADSC (H1), and BTP-hADSC (H2). The rats were sacrificed after 1 and 8 weeks for further study. Runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), osterix (OSX), and bone morphogenetic protein 2 (BMP2) (g/mL) were quantified using immunohistochemistry, while bone tissue volume (BV, mm<sup>3</sup>), bone tissue volume fraction (BF, percentage), and trabecular thickness of bone (TT, mm) were assessed using micro-computed tomography (CT).

### Isolation, Culture, and Differentiation of hADSCs

Fat tissue was collected from a healthy pregnant woman who underwent cesarean section with the previously signed consent form, in accordance with the guidelines of the ethical committee of our hospital (IRB 106/KEH/2018). Isolation and culture of hADSC were carried out until the fourth passage according to Banyard et al.<sup>25</sup> Differentiation was carried out according to Jeon et al.,<sup>26</sup> showing adipogenic and osteogenic lineage, as previously mentioned in our earlier study.<sup>27,28</sup>

### Characterization of hADSC

Characterization of hADSC was analyzed using BD LSR II Flow Cytometer (BD Biosciences). MSC-specific surface markers suggested CD 90, CD 73, and CD 105 were strongly positive expressed

above 95%, whereas CD 14, CD 19, CD 34, CD 45, and human leukocyte antigen (HLA)-DR were found to be negatively expressed below 2%, as previously mentioned in our earlier study.<sup>27,28</sup>

### Bio-Scaffold Preparation

The cadaveric-origin HCG cubical type was obtained from the center of biomaterial and tissue banking in our hospital and inspected before applying as stated earlier.<sup>28</sup> Kasios (Kasios TCP Dental HP; Kasios, L'Union) provided the BTP in the form of granules with a size of 2 to 3 mm, for which the Food and Drug Administration (FDA) issued approval (21 CFR 888.3045). Each HCG or BTP was planted 24 h before implantation on a 24-well microplate (Iwaki), containing  $2 \times 10^6$  cells of hADSC.

### Assay of MTT

An *in vitro* colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) was conducted in H1 and H2 as suggested in the earlier study.<sup>28</sup> It was proven that combination of HCG or BTP with hADSC improved cell viability *in vitro*.<sup>28</sup> The researchers used an inverted microscope (Eclipse TS100, Nikon) and an enzyme-linked immunosorbant assay (ELISA) reader with a 590 nm optical density (ODT).

### Animal Model

Prior to surgery, all experimental models were shaved and measured. Ketamine hydrochloride 5% (Kepro) and xylazine 2% (Kepro) intraperitoneal anesthetic shots were then provided. The second insertion anesthetic was used (2% lidocaine with 1:100,000 epinephrine, 7 mg/kg lidocaine, 5 mg/kg epinephrine, both from Kepro in the Netherlands).

The animals were placed laterally to make the surgery simpler. Lidocaine with adrenaline (0.3 mL) was used as a local anesthetic. In the transition area of the mouth, a 2 cm incision was made. After that, the corresponding muscles and periosteum are dissected. The maxillary bone and the zygoma are fully dissected. To obtain full-thickness bone and build a  $5 \times 5$  mm alveolar defect, fissure burs with constant pace and watering were used. The extracted bone was reattached to the defects (ABG group). In H1 group, HCG-hADSCs were loaded into the defects, while in H2 group, BTP-hADSCs were loaded into the defects. The periosteum was not kept intact in all defects (online Supplemental Material). Fixation was done with Cyrcil 4-0 and local muscle flaps (Ethicon). The skin was sutured with Nylon 5-0 sutures (Ethicon, Brazil).

Meloxicam (2 mg/kg body weight) and ciprofloxacin (10 mg/kg body weight) were given to the animals up to 2 days after surgery. They were provided regular liquid foods for the first week following surgery before going on to a daily diet. The same quantity and form of food were provided for all of the animals. One and 8 weeks after surgery, animals were killed. Tissue specimens were fixed in a 10% buffered formaldehyde solution. Immunohistochemical tests were

conducted to count the number of cells expressing 4 osteogenesis markers: RUNX2, ALP, OSX, and BMP2. Micro-CT scans were used to assess BV, BF, and TT (SkyScan 1173, Bruker).

### Immunohistochemical Analysis

Specimens were decalcified with RapidCal Immuno™ (BBC Biochemical), fixed with 70%, 80%, 90%, and 96%, and absolute dehydrated alcohol, immersed in liquid paraffin at 60°C, and cut with a rotary microtome Accu-Cut® SRM™ 200 (Sakura) with a 1.5 to 2 mm thickness.

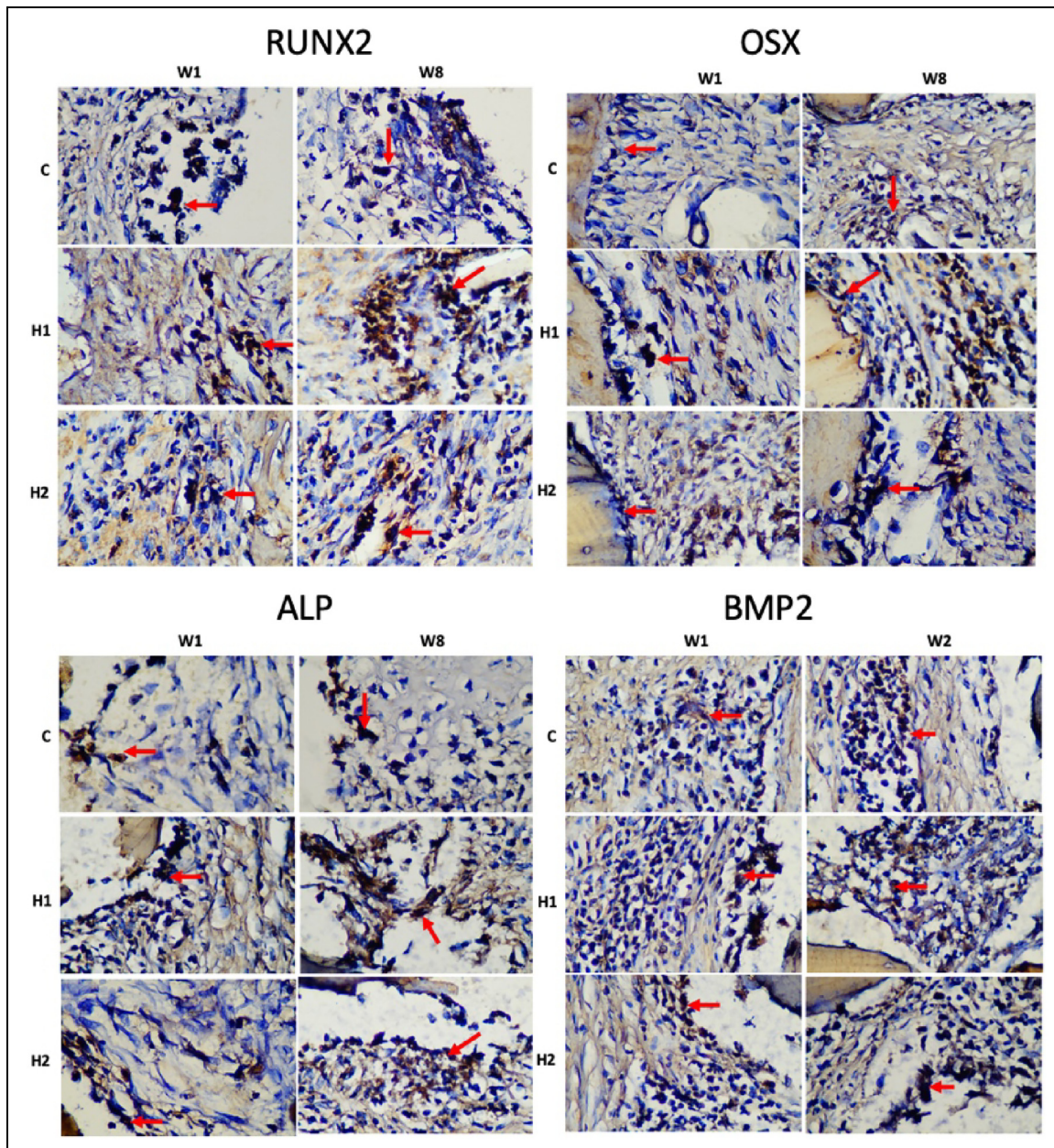
The specimens were rinsed with deionized water 3 times for 5 min per time, then with phosphate-buffered saline (PBS, 70011044, Gibco) for 5 min. Before staining, 200  $\mu$ L of blocking solutions were added for an hour at room temperature. After the blocking solutions were removed, 200  $\mu$ L of RUNX2 primary antibody (sc-101145, Santa Cruz Biotechnology) was applied and incubated overnight at 4°C. The primary antibody was extracted and rinsed using PBS 3 times for 5 min per time. A total of 200  $\mu$ L of secondary goat anti-human IgG—HRP (sc-2004, Santa Cruz Biotechnology) antibodies were applied and incubated at room temperature for half hour. The secondary antibody was extracted and rinsed using tris-buffered saline-tween (TBST, T9039, Sigma-Aldrich) 3 times for 5 minutes per time.

Specimens were then incubated for 10 min with 200  $\mu$ L of chromogenic 3,3'-diaminobenzidine (ab64238, Abcam) before being washed 3 times with PBS for 5 min per time. The specimens then were submerged in deionized water, counterstained with hematoxylin, and rinsed twice in deionized water for 5 min per time. Dehydrated specimens were placed on coverslips. The primary antibodies used for OSX, ALP, and BMP2 were rabbit anti-OSX polyclonal antibody (bs-1110R, Bioss), human anti-mouse monoclonal antibody ALP (A-10) (sc-271431, Santa Cruz Biotechnology), and rabbit anti-BMP2 polyclonal antibody (bs-1012R, Bioss). Secondary antibody goat anti-rabbit IgG—HRP (sc-2004, Santa Cruz Biotechnology) was used for ALP and secondary antibody goat anti-rabbit IgG—HRP (sc-2004, Santa Cruz Biotechnology) was used for OSX and BMP2, respectively.

The average number of positively expressed osteoblast cells inside 20 fields of view was then determined using a microscope at 1000 magnification to evaluate these 4 osteogenesis markers (Figure 1).

### Radiographic Evaluation

Micro-CT scans (SkyScan 1173, Bruker) were conducted on specimens that had been soaked in a 10% buffered formaldehyde fixation solution. The specimens were scanned using a micro-CT scan of the high-energy x-ray. The specimens were mounted on a rotating stand with a rotation phase of 0.20° and a rotation angle of 240° (often referred to as 180+). The x-ray origin has an energy output of 80 kV and current output of 80  $\mu$ A. The x-rays travel through a 1 mm thick aluminum filter and are captured by a 500ms exposure period flat panel



**Figure 1.** Immunohistochemical examination of RUNX2, OSX, ALP, and BMP2 osteogenesis markers. Red arrows represent the osteoblast cells that express markers of osteogenesis. W1 (first week), W8 (eighth week), C (ABG), H1 (HCG-hADSC), and H2 (BTP-hADSC). Abbreviations: ABG, autologous bone graft; ALP, alkaline phosphatase; ANOVA, analysis of variance; BMP2, bone morphogenetic protein 2; BTP, beta tricalcium phosphate; hADSC, human adipose-derived stem cell; HCG, human cancellous freeze-dried graft; OSX, osterix; RUNX2, runt-related transcription factor 2.

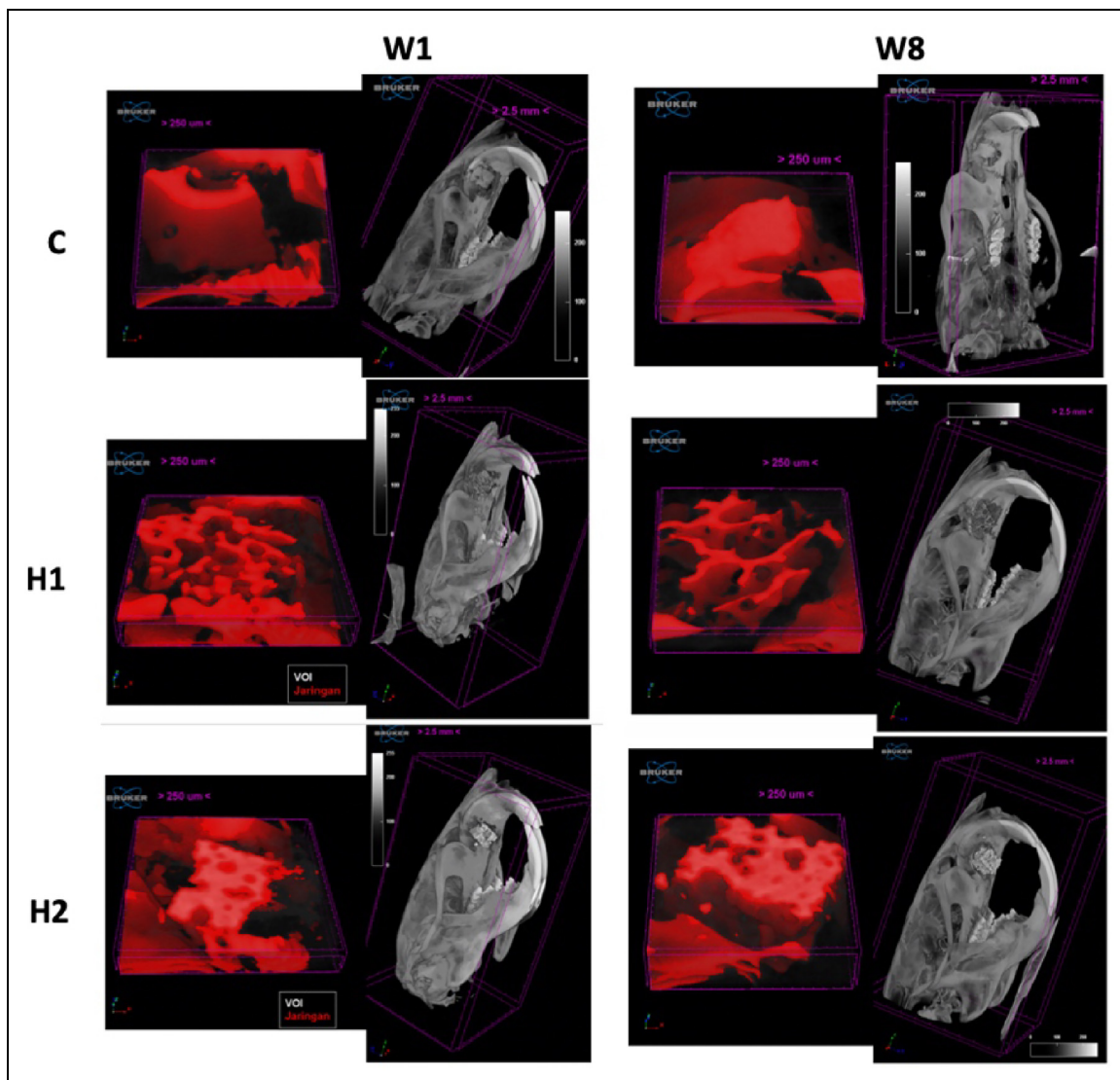
charge-coupled device (CCD) detector. The flat panel CCD was programmed to generate a projection picture by  $1 \times 1$  scaling the camera pixel, resulting in a projection image with a spatial resolution of  $28.15 \mu\text{m}/\text{pixel}$  and image dimensions of  $2240 \times 2240$  (Hi-Res scan mode). The random movement was set to 10 to eliminate circle artifacts and the frame averaging was set to 10 to decrease random noise.

The GPUReconServer kernel based on Feldkamp backpropagation was used to perform three-dimensional reconstruction with the NRecon program. The specimens were then analyzed

by measuring BV, BF, and TT using the previously stated description (Figure 2).

### Statistical Analysis

The information was held in the context of a mean and standard deviation (Table 1). The variance in variables among groups at 1 and 8 weeks after surgery was assessed using a one-way ANOVA test with post hoc analysis least significant difference (LSD) (Table 2). It was decided that a statistical equivalent of *P*



**Figure 2.** Micro-CT radiological examinations at the first (W1) and eighth (W8) weeks. The  $5 \times 5$  mm size of the alveolar bone critical defect provides treatment with ABG (C), HCG-hADSC (H1), and BTP-hADSC (H2). Abbreviations: ABG, autologous bone graft; BTP, beta tricalcium phosphate; hADSC, human adipose-derived stem cell; HCG, human cancellous freeze-dried graft.

$<.05$  was significant. For the analysis, the Statistical Package for Social Science was used (IBM Corp. Released 2016, IBM SPSS Statistics for Macintosh, Version 24.0: IBM Corp).

## Results

The result of immunohistochemical examination was shown in Figure 3. RUNX2 was substantially different among groups ( $P = 0.001$ ) at 1 week after surgery (Table 2), and post hoc analysis indicated that H1 and H2 had higher RUNX2 than C ( $P = 0.001$ ). RUNX2 was substantially different among groups ( $P = .000$ ) at 8 weeks after surgery (Table 2), and post hoc analysis indicated higher RUNX2 in H1 and H2 than C ( $P = .000$ ) and H1 than H2 ( $P = .027$ ).

OSX was substantially different among groups ( $P = .003$ ) at 1 week after surgery (Table 2), and post hoc analysis revealed that H1 ( $P = 0.002$ ) and H2 ( $P = 0.003$ ) had higher OSX than C. OSX was substantially different among groups ( $P = .002$ ) at 8 weeks after surgery (Table 2), and post hoc analysis indicated that H1 ( $P = .001$ ) and H2 ( $P = .004$ ) had higher OSX than C.

ALP was substantially different among groups ( $P = .005$ ) at 1 week after surgery (Table 2), and post hoc analysis revealed that H2 had higher ALP than C ( $P = .003$ ) and H1 ( $P = .002$ ). ALP was substantially different among groups ( $P = .008$ ) at 8 weeks after surgery (Table 2), and post hoc analysis indicated that H1 ( $P = .001$ ) and H2 ( $P = .004$ ) had higher ALP than C.

BMP2 was substantially different among groups ( $P = .002$ ) at 1 week after surgery (Table 2), post hoc analysis indicated that H2 had higher BMP2 than C ( $P = .000$ ) and H1 ( $P = .013$ ). BMP2 was



**Table 1.** The Average Variable of Each Group.

Mean $\pm$ standard deviation		C	H1	H2
RUNX2	W1	7.60 $\pm$ 1.09	11 $\pm$ 1.87	11.18 $\pm$ 1.20
	W8	11.25 $\pm$ 1.78	18.43 $\pm$ 1.00	16.25 $\pm$ 1.73
OSX	W1	7.92 $\pm$ 1.80	11.58 $\pm$ 1.59	11.25 $\pm$ 1.57
	W8	11.37 $\pm$ 1.97	16.01 $\pm$ 1.18	15.11 $\pm$ 2.38
ALP	W1	8.93 $\pm$ 1.10	10.08 $\pm$ 2.29	13.17 $\pm$ 2.16
	W8	11.85 $\pm$ 2.10	17.25 $\pm$ 3.21	15.2 $\pm$ 2.30
BMP2	W1	8.42 $\pm$ 1.19	10.25 $\pm$ 0.69	13.42 $\pm$ 3.06
	W8	12.15 $\pm$ 0.57	19.01 $\pm$ 3.10	14.78 $\pm$ 1.31
Bone tissue volume	W1	6.95 $\pm$ 1.83	5.48 $\pm$ 1.70	7.58 $\pm$ 0.61
	W8	5.59 $\pm$ 2.42	5.79 $\pm$ 1.49	9.66 $\pm$ 3.02
Bone tissue volume fraction	W1	33.02 $\pm$ 10.14	23.95 $\pm$ 7.67	34.27 $\pm$ 3.99
	W8	25.16 $\pm$ 13.36	24.75 $\pm$ 6.89	35.32 $\pm$ 5.30
Mean trabecular Thickness of bone	W1	0.35 $\pm$ 0.05	0.29 $\pm$ 0.04	0.42 $\pm$ 0.04
	W8	0.32 $\pm$ 0.06	0.30 $\pm$ 0.05	0.44 $\pm$ 0.03

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; RUNX2, runt-related transcription factor 2; OSX, osterix.

**Table 2.** Significance Difference Between Groups at 1 Week and 8 Weeks After Surgery ( $P < .05$ ).

One-way ANOVA	M1		M8	
	Significance (two-tailed)	Post hoc analysis	Significance (two-tailed)	Post hoc analysis
RUNX2	.001	H1 > C (.001) H2 > C (.001)	.000	H1 > C (.000) H2 > C (.000) H1 > H2 (.027)
OSX	.003	H1 > C (.002) H2 > C (.003)	.002	H1 > C (.001) H2 > C (.004)
ALP	.005	H2 > C (.003) H2 > H1 (.002)	.008	H1 > C (.001) H2 > C (.004)
BMP2	.002	H2 > C (.000) H2 > H1 (.013)	.000	H1 > C (.000) H2 > C (.036) H1 > H2 (.002)
Bone tissue volume	.072		.016	H2 > C (.010) H2 > H1 (.013)
Bone tissue volume fraction	.069		.112	
Mean trabecular thickness of bone	.000	H2 > C (.008) C > H1 (.031)	.000	H2 > C (.001) H2 > H1 (.000)

Abbreviations: ALP, alkaline phosphatase; ANOVA, analyses of variance; BMP2, bone morphogenetic protein 2; RUNX2, runt-related transcription factor 2; OSX, osterix.

substantially different among groups ( $P = .000$ ) at 8 weeks after surgery (Table 2), post hoc analysis indicated higher BMP2 in H1 ( $P = .000$ ) and H2 than C ( $P = .036$ ) and H1 than H2 ( $P = .002$ ).

The result of micro-CT examination was shown in Figure 3. BV was also similar at 1 week after surgery among groups (Table 2). BV was substantially different ( $P = .016$ ) at 8 weeks after surgery among groups (Table 2), post hoc analysis indicated higher BV in H2 than C ( $P = .010$ ) and H1 ( $P = .013$ ).

BF was also similar at 1 and 8 weeks after surgery among groups (Table 2).

TT was substantially different ( $P = .000$ ) at 1 week after surgery among groups (Table 2), post hoc analysis indicated higher TT in H2 than C ( $P = .008$ ) and C than H1 ( $P =$

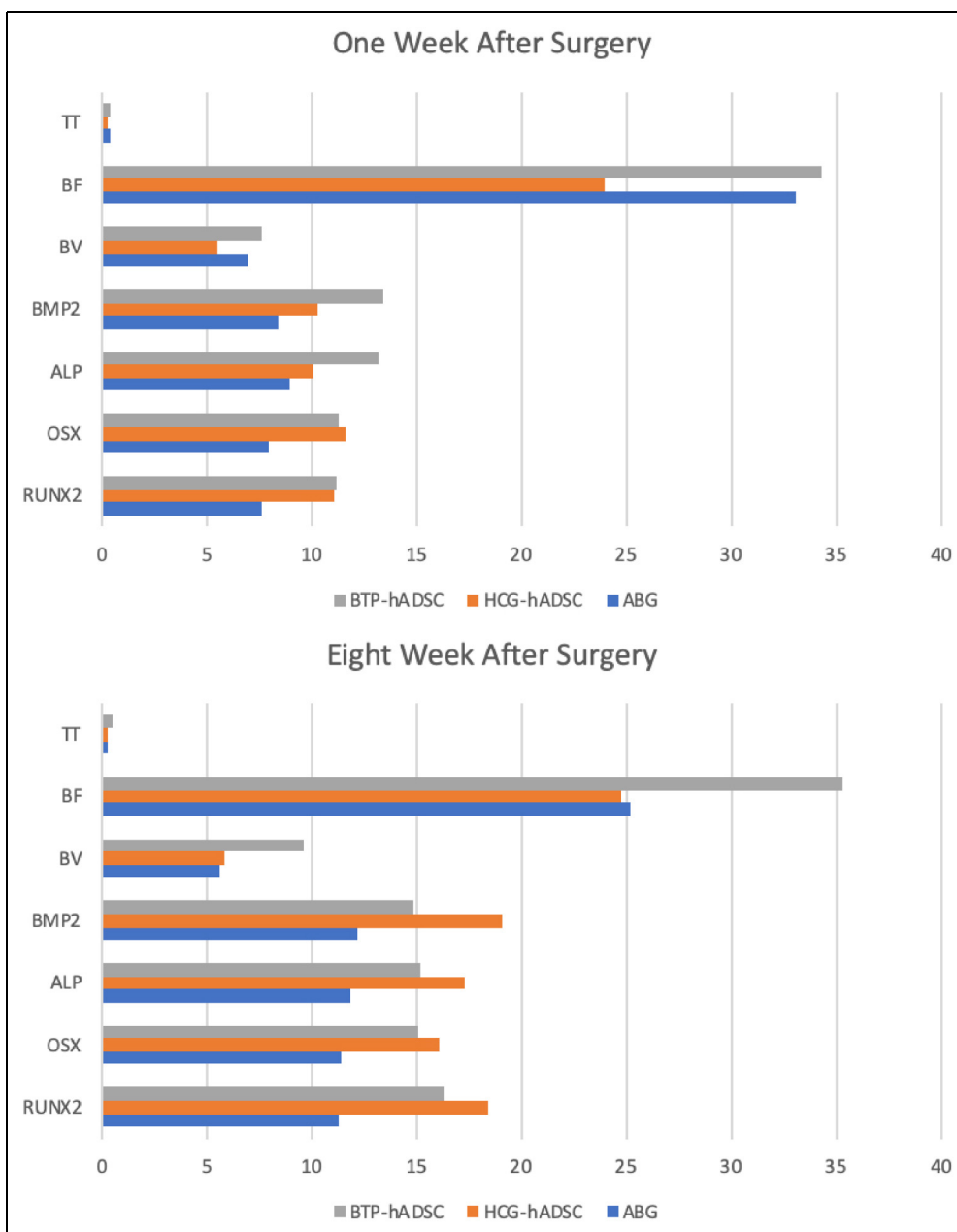
.031). TT was substantially different ( $P = .000$ ) at 8 weeks after surgery among groups (Table 2), post hoc analysis indicated higher TT in H2 than C ( $P = .001$ ) and H1 ( $P = .000$ ).

There were no postoperative infections in all groups.

## Discussion

The activity of BMP2, an upstream osteogenesis pathway,<sup>29–33</sup> was elevated, accompanied by the signaling of RUNX2, a pre-osteoblast indicator that stimulates OSX signaling,<sup>29,34</sup> and then the activity of ALP,<sup>35</sup> an osteoblast marker.

At 1 week after surgery, Yu et al.<sup>36</sup> found that there was a higher rise in ALP in BTP–rabbit bone marrow mesenchymal stem cell (BMMSC)–rabbit platelet-rich plasma (PRP) relative



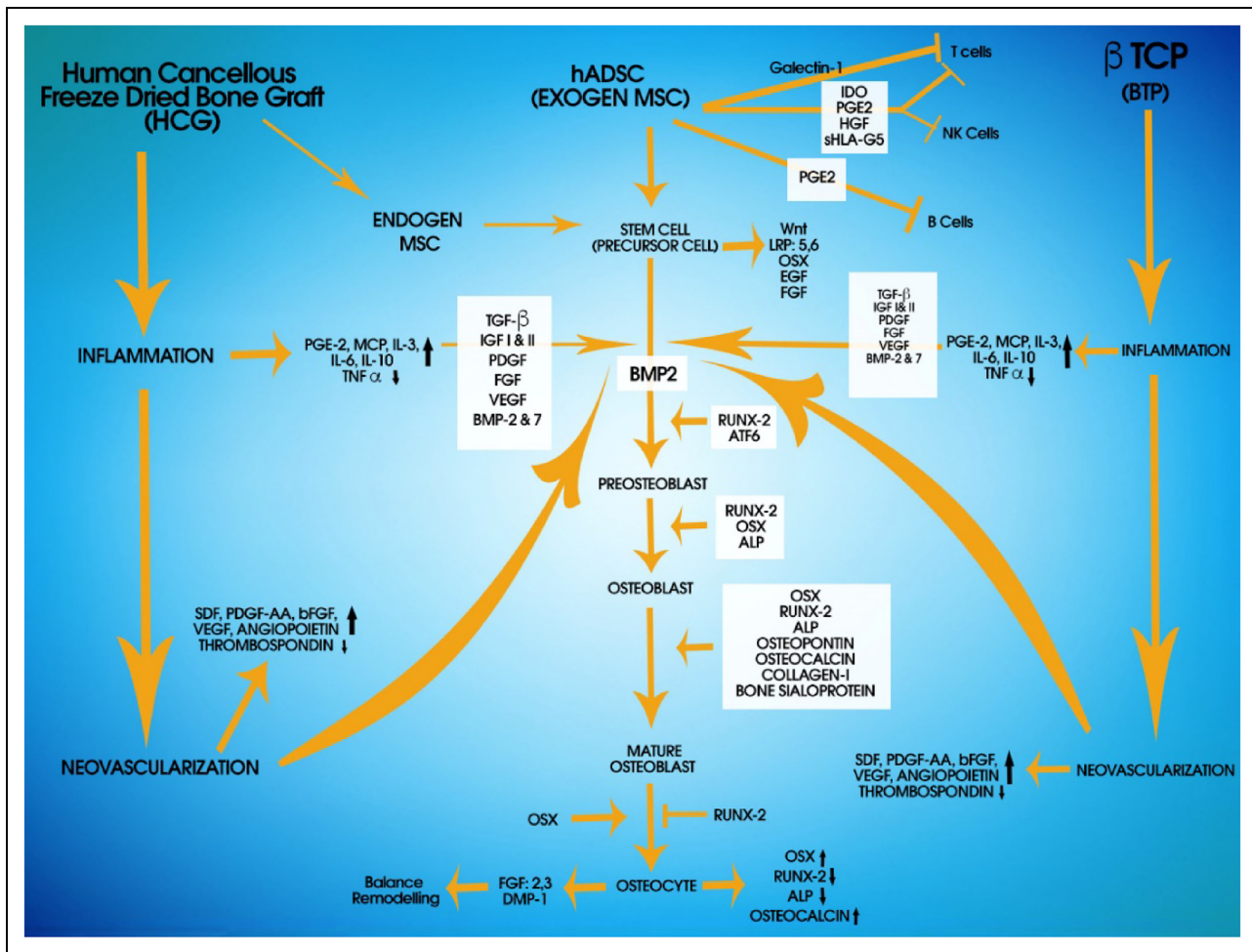
**Figure 3.** Diagram of examination results 1 and 8 weeks after surgery.

Abbreviations: ABG, autologous bone graft; ALP, alkaline phosphatase; ANOVA, analyses of variance; BF, bone tissue volume fraction; BMP2, bone morphogenetic protein 2; BTP, beta tricalcium phosphate; BV, bone tissue volume; hADSC, human adipose-derived stem cell; HCG, human cancellous freeze-dried graft; OSX, osterix; RUNX2, runt-related transcription factor 2; TT, trabecular thickness of bone.

to BTP-rabbit BMMSC on rabbit radius bone. Saskianti et al.<sup>37</sup> conducted a study on rat alveolar defects and found that the expression of OPG, RUNX2, transforming growth factor beta (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), ALP, osteocalcin, and osteopontin was significantly higher in the human exfoliated deciduous teeth (SHED) and carbonate apatite (CAS) compared to the CAS group alone.

At 1 week after surgery, we found RUNX2, OSX was higher in HCG-hADSC and BTP-hADSC than ABG. ALP and BMP2 was higher in BTP-hADSC than ABG and HCG-hADSC. As

we know BTP had high biocompatibility, osteoconductivity, and accelerated bone forming when exposed to bone tissue,<sup>11,12</sup> administration of hADSC further accelerates the osteoinduction and osteogenesis ability of BTP, therefore increase of RUNX2, OSX, ALP, and BMP2 expression were found in BTP-hADSC. HCG is a human cancellous bone graft, where a freeze-dried process was carried out for the purpose of controlling the transmission of infectious agents. This freeze-drying process could reduce the osteoinduction and osteoconduction ability of HCG.<sup>8</sup> Therefore, only



**Figure 4.** Osteoblastogenesis starts with the expression of growth factors, one of which is BMP2, which induces RUNX2 into the preosteoblast phase. Overexpression of BMP2 and RUNX2 during this process will cause OSX for osteoblast development, and as osteoblasts mature, they will express collagen type I and ALP. Mature osteoblasts can differentiate into osteocytes. BMP2 expression can lead to the expression of RUNX2, OSX, and ALP, while RUNX2 expression can lead to the expression of OSX and ALP. HCG and BTP have osteoconduction potential, which plays a role in the inflammatory mechanism, that is, growth factor secretion and neovascularization, but only HCG has osteoinduction potential, which plays a role in the involvement of endogenous MSC. The combination of HCG or BTP with hADSC, which are exogenous mesenchymal stem cells, will increase new bone formation by supplying osteogenic components of mature cells, which will also increase the ability of scaffold osteoinduction and osteoconduction, which will also indirectly play a role in immunomodulation and immunosuppression such that there is no rejection in the recipient region.

Abbreviations: ABG, autologous bone graft; ALP, alkaline phosphatase; ANOVA, analyses of variance; BMP2, bone morphogenetic protein 2; BTP, beta tricalcium phosphate; hADSC, human adipose-derived stem cell; HCG, human cancellous freeze-dried graft; MSC, mesenchymal stem cell; OSX, osterix; RUNX2, runt-related transcription factor 2.

RUNX2 and OSX expression was higher in HCG–hADSC compare to ABG, in accordance to Bruderer et al.<sup>38</sup> stated that the addition of mesenchymal stem cells boosts RUNX2 and OSX expression in the early phase.

At 8 weeks after surgery, we found the highest RUNX2 and BMP2 expression in HCG–hADSC followed by BTP–hADSC then ABG. OSX and ALP were higher in HCG–hADSC and BTP–hADSC than C. This proved that there was an acceleration of the osteogenesis, osteoinduction, and osteoconduction ability of HCG at week 8, which may be due to the effect of hADSC administration, therefore increase of RUNX2, OSX, ALP, and BMP2 expression were found in HCG–hADSC, even more RUNX2 and BMP2 expression was higher than BTP–hADSC,

making HCG–hADSC superior among other groups in immunohistochemical analysis followed by BTP–hADSC then ABG.

At 1 week after surgery, Seebach et al.<sup>39</sup> observed a substantial improvement of vascularization in rat femoral bone in the BTP–endothelial progenitor cells (EPC) and BTP–human BMMSC (hBMMSC)–EPC compare to ABG, BTP only, and BTP–hBMMSC. We found BV and BF were similar among groups, but the highest TT was seen in BTP–hADSC followed by ABG then HCG–hADSC. This could be due to the fact that it was still in the early stages and the state of the bone implantation had not changed. It also described the initial condition of the scaffold where BTP had the highest trabecular thickness.

At 8 week after surgery, Seebach et al.<sup>39</sup> observed the greatest bone structure and vascularization of rat femoral bone in the BTP–hBMMSC–EPC group, compare to BTP–hBMMSC, ABG, BTP–EPC, and BTP only groups. Zhou et al.<sup>40</sup> found that the BTP–rabbit BMMSC–rabbit MSC-derived endothelial cells had the greatest new bone-forming and marrow cavity regeneration in the ulnar bone of rabbits, according to x-ray analysis relative to the untreated, BTP only, and BTP–rabbit BMMSC. Park et al.<sup>41</sup> found higher BV of rat tibial defect in poly lactic-glycolic acid (PLGA)–ADSC group compared to PLGA-only group. Yu et al.<sup>36</sup> found that there was bonier callus in the x-ray study and more thorough bone structure in the histological study in BTP–rabbit BMMSC–rabbit PRP relative to BTP–rabbit BMMSC on rabbit radius bone. Jahanbin et al.<sup>42</sup> found maximum bone formation of rat alveolar defects in ABG followed by collagen matrix-hDPSC, then collagen matrix-only group. Amaral et al.<sup>24</sup> found no differences in the alveolar bone of rats utilizing micro-CT analysis in MDSC– $\alpha$ TCP relative to ABG. Orbay et al.<sup>43</sup> found no differences in calvarial defect of rats among groups consisting hydroxyapatite (HA)–poly lactide-co-glycolide (PLG), HA–PLG–ADSC, HA–PLG–ADSC-derived endothelial cells and HA–PLG–ADSC-derived osteoblast.

Soliman et al.<sup>44</sup> found that the successful rate of the alveolar clef repair using ABG was 77.8%, ADSC–ABG was 90%, and ADSC–demineralized bone matrix (DBM) was 50%, but there was no statistically significant difference between the groups. ADSC was harvested from abdominal fat at the end of surgery and administered 1 week postoperatively in the outpatient clinic. Shahnaseri et al.<sup>45</sup> found no differences in radiographic density in bilateral canine alveolar defects treated with ABG on one side and ADSC–HA–BTP on the other.

At 8 weeks after surgery we found that BV was higher in BTP–hADSC than ABG and HCG–hADSC, BF was similar among groups and TT was higher in BTP–hADSC than ABG and HCG–hADSC. This proved that BTP–hADSC had superior BV and TT, among other groups, and proved no differences between HCG–hADSC and ABG in the alveolar defect of rats utilizing micro-CT analysis.

As previously stated, both HCG and BTP have osteoconductive but no osteogenic features, whereas only HCG possesses osteoconductive features.<sup>9,10</sup> Following the implantation of HCG or BCP in the site of the defect, inflammation occurs, resulting in the development of inflammatory mediators such as prostaglandin E-2 (PGE-2), monocyte chemoattractant proteins, interleukin 3 (IL-3), IL-6, IL-10, and tumor necrosis factor alpha.<sup>46</sup> Both osteoconductors will play a role in the angiogenesis and neovascularization processes, which are influenced by the following factors: stromal cell-derived growth factor, platelet-derived growth factor (PDGF)-AA, basic fibroblast growth factor, angiopoietin, and thrombospondin.<sup>47,48</sup> Growth factors such as TGF- $\beta$ , insulin-like growth factor I (IGF I), IGF II, PDGF, VEGF, BMP2, and BMP7 have been linked to osteogenesis.<sup>49</sup> The osteoinduction potential of HCG will contribute to the involvement of endogenous MSC, which will be more effective with the addition of

exogenous MSC derived from hADSC, resulting in an osteogenic component to the combination. It will encourage new bone formation by delivering mature cell osteogenic components.

MSC also perform an indirect function in immunomodulation and immunosuppression, ensuring that there is no rejection in the recipient region. The MSC-secreted soluble molecules such as indoleamine 2, 3-dioxygenase (IDO), PGE-2, and soluble HLA-G5 may reduce T and natural killer (NK) cell activities, but galectin-1 inhibits T cells but not NK cells. Furthermore, by suppressing dendritic cells and encouraging the growth of regulatory T cells, MSC may indirectly cause immunosuppression. Furthermore, MSC may interact with T and NK cells directly via cell contact-dependent interaction mechanisms that are still completely unexplored.<sup>50</sup>

Osteogenic features will stimulate osteoblastogenesis. The expression of growth factors, one of which is BMP2, will first upregulate RUNX2, making cells more receptive to BMP2, which will then upregulate RUNX2 and other downstream mediators, inducing osteoblastogenesis.<sup>29,30</sup> RUNX2 and OSX are 2 osteogenic master transcription factors that are stimulated by BMP2.<sup>51</sup> Overexpression of RUNX2 induces OSX, indicating that OSX is downstream of RUNX2.<sup>29,34</sup> RUNX2 expression must be reduced to allow for further maturation.<sup>38</sup> Overexpression of BMP2 and RUNX2 causes OSX for osteoblast development, and when osteoblasts mature, they express collagen type 1 and ALP.<sup>35,38</sup> Mature osteoblasts have the ability to develop into osteocytes.

All of the above had proven that exogenous hADSCs enhanced the effectiveness of BTP and HCG for osteogenesis, osteoconduction, and osteoinduction (Figure 4). By administering hADSC to BTP and HCG, making it comparable even superior to ABG, which is the preferred method for managing bone defects, but it requires around 4 months for osteoblast maturation until cells make up bone matrix.<sup>52</sup> Although our research centered on 1 and 8 weeks, it would be important to expand the period of examination to 12 and 16 weeks to define the late stage of osteoblast maturation activity, where, in both immunohistochemical and micro-CT analysis final result, HCG–hADSC may be the most superior of all, or possibly the other way around.

There are many existing clinical studies where stem cells have been used for bone regeneration, but only few studies used assessment parameters similar to ours. There are many existing clinical studies where ADSC has been used for bone regeneration, but only a few studies where ADSC has been specifically used for alveolar bone regeneration. Hopefully in the future, there will be more similar studies so that there will be more and more discussion regarding the use of ADSCs in alveolar bone regeneration.

## Conclusion

Exogenous hADSCs enhanced the effectiveness of HCG and BTP to accelerate osteogenesis, osteoconduction, and osteoinduction. HCG–hADSC was superior among others followed by BTP–hADSC then ABG in the expression of RUNX2, OSX, ALP, and BMP2. BTP–hADSC was superior among

others in bone formation, followed by HCG-hADSC and ABG, which had comparable effectiveness.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


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### Ethical Approval

Ethical approval was obtained from the Ethical Committee of Airlangga University Hospital (IRB 106/KEH/2018).

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### Supplemental Material

Supplemental material for this article is available online.

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