Reviewer Invitation (first manuscript submission)

1 message

European Journal of Dentistry <ejd@manuscriptmanager.net> Mon, Jun 20, 2022 at 6:15 AM
To: tania-s@fkg.unair.ac.id

Manuscript: EJD-2022-5-31 - (2136) - The Osteogenesis Mechanisms of Dental Alveolar Bone Socket Induction with Hydroxyapatite Bovine Tooth Graft: An Expression of BMP-2, RUNX2, OSX, OCN, Osteoblast, and Osteoclast in Animal Experimental Rattus Norvegicus Strain Wistar
Date submitted: 2022-06-02

Dear Dr. Saskianti

The above manuscript has been submitted to the European Journal of Dentistry. Given your expertise in this field, I am kindly asking if you would be willing to review this manuscript.

It is the policy of the journal to ensure that the submission process is quick and efficient and, therefore, we schedule only 14 days for the reviewing process. If you agree to review this manuscript, we assume that you accept this condition and have the available time to complete the task within the stipulated time. It would greatly assist us if you could respond as soon as possible.

Please click the link below and then agree or decline to review this manuscript. If you decline, you will have the opportunity to suggest another potential reviewer.

I do hope that the journal can take advantage of your expertise.

Thank you,

Sincerely,

Dr. Nejdet Adanir, DDS, PhD.
Editor-in-Chief, European Journal of Dentistry
Associate Professor, Department of Restorative Dentistry
College of Dentistry, King Faisal University
Al Ahsa, KSA, 31982
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ABSTRACT: Abstract
Objective : This study to determine the mechanism of bone healing of the alveolar bone socket post dental extraction of Wistar rats, after administration of a bovine tooth graft (Hydroxyapatite Bovine Tooth Graft = HAp-BTG).
Materials and Methods : A total of 50 Wistar rats were randomly selected into two groups, control and treatment, and into five sub groups, on days 3, 7, 14, 21, and 28. The post extraction socket was filled with PEG (Polyethylene glycol) as the control and PEG + HAp-BTG as the treatment group. On days 3, 7, 14, 21, and 28, Wistar rats were sacrificed, mandibles were taken, paraffin blocks were made, cut 4 microns thick, and made into glass preparations for microscopic examination. The variable analysis was performed by staining HE (Hematoxylin-Eosin) for osteoblasts and osteoclasts and immunohistochemistry for RUNX2, OSX, OCN, BMP-2. The expressed cell count per microscope field were analysed.
Statistical Analysis : All data were tabulation and analyzed using SPSS Version 25, with a significance of 0.05. We did Analysis between the control versus treatment group and between the time series.
Results : In general, the number of cell expressions in the treatment group was significantly higher, but the lower significantly of OC. The increased peak of variables occurred on day 14 for BMP-2, RUNX2, and OCN, on day 7 for OSX, while OB was significantly increased on day 21 and remained until day 28. The decrease of OC cells occurred on day seven and remained low until 28 days.
Conclusion : It was concluded that the administration of HAp-BTG can accelerate alveolar bone healing as indicated by increased expression of BMP-2, RUNX2, OSX, OCN, number of osteoblasts, and decreased osteoclasts.

Keywords : Hydroxyapatite Bovine tooth graft, Osteogenesis, Osteoblast, Medicine

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Attention: Dr. Saskianti

Automatic notification:

This is an automatic notification to inform you that your evaluation of the above manuscript that you are kindly undertaking for the journal is due in a few days. Your valuable contribution to the journal is much appreciated and we do hope you find the time to return your report before the deadline.

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Attention: Dr. Saskianti

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tania saskianti <tania-s@fkg.unair.ac.id>

EJD-2022-5-31 - (2136) Thank you for your review

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European Journal of Dentistry <ejd@manuscriptmanager.net> Mon, Jul 4, 2022 at 6:42 PM
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Attention: Dr. Saskianti

Manuscript: EJD-2022-5-31 - (2136) - The Osteogenesis Mechanisms of Dental Alveolar Bone Socket Induction with Hydroxyapatite Bovine Tooth Graft: An Expression of BMP-2, RUNX2, OSX, OCN, Osteoblast, and Osteoclast in Animal Experimental Rattus Norvegicus Strain Wistar

Date submitted: 2022-06-02

Dear Dr. Saskianti

An editorial decision has now been made for the above manuscript that you have kindly reviewed for European Journal of Dentistry.
Please find below a copy of the decision letter sent to the authors.

We would like to take this opportunity to thank you for your invaluable assistance and hope that we can call on your expertise again in the near future.

Sincerely,

Dr. Nejdet Adanir, DDS, PhD.
Editor-in-Chief, European Journal of Dentistry
Associate Professor, Department of Restorative Dentistry
College of Dentistry, King Faisal University
Al Ahsa, KSA, 31982
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Date submitted: 2022-06-02

Dear Dr.

Thank you very much for submitting the above manuscript to European Journal of Dentistry.

Your manuscript has now been evaluated by external reviewers and members of the editorial board. While we certainly find merit in your paper, a number of points of criticism were raised and we therefore regret to inform you that your manuscript is not acceptable for publication in its present form. If you feel that you can adequately address the criticisms outlined in the reviewer reports below, we would be willing to reconsider a thoroughly revised version of your manuscript.

If you decide to resubmit your manuscript, please provide point-by-point answers to the reviewer criticisms in the text field provided during the online resubmission process and highlight any revisions made in your manuscript document.

We look forward to receiving the revised version within three (3) weeks and thank you, again, for submitting your

manuscript to European Journal of Dentistry.

Sincerely,

Dr. Nejdet Adanir, DDS, PhD.
Editor-in-Chief, European Journal of Dentistry
Associate Professor, Department of Restorative Dentistry
College of Dentistry, King Faisal University
Al Ahsa, KSA, 31982
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Reviewer 1 report:
Comments to authors
- Title and Abstract was well-structured, accurate, inclusive, and well-presented prompt to attract potential readers.
- Kindly put more clear statement of the problem in the introduction.
- Material and Methods. Explain more about randomization.
- Result was well written and strong.
- Figure and tables are clear and legible.
- Avoid repeating results in discussion.
- It is better to discuss controversial articles in discussion part.
- Suggestions for future research were not mentioned.
- Limitations of the study were not clearly discussed.
- Conclusions were well written.

Reviewer 2 report:
Comments to authors
Comments:
Dear Author,

I would like add some comments regarding your manuscript “The Osteogenesis Mechanisms of Dental Alveolar Bone Socket Induction with Hydroxyapatite Bovine Tooth Graft: An Expression of BMP-2, RUNX2, OSX, OCN, Osteoblast, and Osteoclast in Animal Experimental Rattus Norvegicus Strain Wistar

However, there are several unclear meaning in your study.

1. Introduction:
a. The title too long
a. Please state your hypothesis and the aim of this study clearly.
c. Why choose the Polyethylene glycol as a comparison?

2. Material and Methods section:
please add more information, specific and clearly method and material that had used in this study.
How to make a HAp-PEG combination mixture?

3. Result:
add figure 2 & 8 IHC : arrow yellow

5. Discussions:
How implication of the study and the next study suggestion?

6. Conclusion: make it short and clear, and answer the hypothesis

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Editor-in-Chief comments:

Cross-referencing: We would like to emphasize that we attach great importance to cross-referencing recent papers on the same topic in the European Journal of Dentistry. Therefore, it would be highly appreciated if you would check the last 5 years of the European Journal of Dentistry and cite references relevant to your article.
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Keywords:	Hydroxyapatite Bovine tooth graft, Medicine, Osteogenesis
Type:	Original/Research Article

The Osteogenesis Mechanisms of Dental Alveolar Bone Socket Post Induction with *Hydroxyapatite* Bovine Tooth Graft: An Expression of BMP-2, RUNX2, OSX, OCN, Osteoblast, and Osteoclast in Animal Experimental *Rattus Norvegicus Strain Wistar*

Abstract **Objective** This study to determine the mechanism of bone healing of the alveolar bone socket post dental extraction of Wistar rats, after administration of a bovine tooth graft (*Hydroxyapatite* Bovine Tooth Graft = *HAp*-BTG).

Materials and Methods A total of 50 Wistar rats were randomly selected into two groups, control and treatment, and into five sub groups, on days 3, 7, 14, 21, and 28. The post extraction socket was filled with PEG (Polyethylene glycol) as the control and PEG + *HAp*-BTG as the treatment group. On days 3, 7, 14, 21, and 28, Wistar rats were sacrificed, mandibles were taken, paraffin blocks were made, cut 4 microns thick, and made into glass preparations for microscopic examination. The variable analysis was performed by staining HE (Hematoxylin-Eosin) for osteoblasts and osteoclasts and immunohistochemistry for RUNX2, OSX, OCN, BMP-2. The expressed cell count per microscope field were analysed.

Statistical Analysis All data were tabulation and analyzed using SPSS Version 25, with a significance of 0.05. We did Analysis between the control versus treatment group and between the time series.

Results In general, the number of cell expressions in the treatment group was significantly higher, but the lower significantly of OC. The increased peak of variables occurred on day 14 for BMP-2, RUNX2, and OCN, on day 7 for OSX, while OB was significantly increased on day 21 and remained until day 28. The decrease of OC cells occurred on day seven and remained low until 28 days.

Conclusion It was concluded that the administration of *HAp*-BTG can accelerate alveolar bone healing as indicated by increased expression of BMP-2, RUNX2, OSX, OCN, number of osteoblasts, and decreased osteoclasts.

Keywords

- *Hydroxyapatite* Bovine tooth graft
- Osteogenesis
- Osteoblast
- Medicine

1. Introduction

Endodontic surgery is part of the field of endodontics through hemisection procedures. This procedure involves the alveolar bone socket, which will impact the defect in the alveolar bone and the peri radicular tissue of the surrounding teeth. In general, soft tissue and hard tissue will be traumatized during tooth extraction. Trauma that occurs due to tooth extraction will experience a natural healing process by going through 3 stages of the wound healing stages: inflammation, proliferation, and remodeling. In the bone healing processes, there is the involvement of osteoblasts and osteoclasts.[1,2]

The reconstruction of bone defects is still a challenge for endodontists in the field of endodontic surgery. It is because the healing process is often interrupted or even failed. Ideally, the success of treatment expects to occur new bone regeneration. In the clinical practice for improving the bone healing process, commonly using a substitute material, namely bone graft, is widely used in regenerative bone procedures.[3-5] Classification of grafting materials includes autograft, allograft, alloplastic graft, and xenograft. The xenogenic graft material, bovine hydroxyapatite (*HAp*), is commonly used in dentistry. This graft has osteoconductive and osteoinductive properties. It allows new bone tissue to grow in the spaces between its mineral particles.[6,7]

Xenografts have commonly known as an alternative to fillers scaffolds. They are relatively easy to use in the maintenance of dental alveolar bone sockets and facilitate bone formation and promote wound healing.[7] Many current studies have developed hydroxyapatite as a bone graft material.[8-10] Still, so far, there has been no research on

the use of hydroxyapatite bovine tooth graft (*HAp*-BTG), a graft material derived from bovine teeth.

Bovine teeth have inorganic and organic components that resemble human teeth components.[11] The organic ingredients of dentin and cementum include type I collagen and various growth factors such as bone morphogenic proteins (BMPs). Type I collagen occupies approximately 90% of the tissue's organic content, and the rest is a non collagenous protein (NCP), biopolymers, citrate, lactate, lipids, and others. NCPs are a specific non collagenous protein in dentin. It includes osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), and osterix (OSX). The others NCPs are runt related transcription factor 2 (RUNX2) and dentin phosphoprotein (DPP).[12-15]

BMP has an essential role in embryonic development, including brain and bone formation. BMP-2 increases osteocalcin (OCN) expression, and its short term expression is required sufficient to induce bone formation. BMP-2 also has a unique role in post natal bone formation.[16] The expression of the OCN gene increased the expression of osterix (OSX) and RUNX2 as a typical marker of osteoblast function. Different from RUNX2, OCN is a marker of end stage differentiation.[17] RUNX2, as a preosteoblast, is a transcription factor closely related to the osteoblast phenotype.[33] OSX is a gene transcription factor identified at the end of the differentiation of preosteoblast cells to osteoblast cells. OSX regulates late stage osteogenesis and inhibits chondrogenesis.[18]

Many studies have succeeded in using bone graft material from bovine bone containing micro sized hydroxyapatite. It limited information the study of hydroxyapatite bovine tooth graft (*HAp*-BTG) material, especially for the osteogenesis process of alveolar

bone sockets. The bovine bone xenograft study showed the significant expression of RUNX2, type I collagen, alkaline phosphatase, and osteocalcin within 14 and 28 days.[15]

This study will explore the cellular and sub cellular mechanisms of osteogenesis by applying *HAp*-BTG graft material in dental socket post dental extraction in Wistar rat. The core factors were used as indicators, namely BMP-2, RUNX2, OSX, OCN, osteoblasts, and osteoclasts.

2. Materials and Methods

This research is an experimental laboratory animal using a post test only control group design. This research has received ethical approval from the Ethics Committee, Faculty of Dental Medicine, Universitas Airlangga (348/HRECC.FODM/VII/2020). We conducted the study at the Biochemistry Laboratory, Faculty of Medicine, Universitas Airlangga.

The experimental animals were *Rattus norvegicus strain Wistar* aged 12-14 weeks, male with a body weight of 250-300 grams, healthy rats, no tooth decay or defects in the whole body.[15,19] The material used is hydroxyapatite bovine tooth graft (*HAp*-BTG) powder type (particle size is about 3.5 μm) which was sterilized by gamma rays at BATAN (National Atomic Energy Agency = National Atomic Energy Center) Jakarta, Indonesia. Preparation of *HAp*-BTG by making a combination mixture of PEG (Polyethylene glycol) 400 and PEG 4000 made with an 80% : 20% ratio.[20]

Total 50 Wistar rats were randomly selected into two groups (control and treatment with 25 each), and every group consisted of 5 sub groups, 3, 7, 14, 21, and 28 days. Anesthesia using a combination of xylazine and ketamine with a ratio of 1:1

intramuscular injection. Termination with an anesthetic injection in the right posterior femoral region. Lower left incisor teeth extraction using incisor extraction forceps. The apical site of the post extraction socket was filled with PEG in group I (control) and PEG + *HAp*-BTG as group II (treatment) as much as 0.1 ml using a syringe. Furthermore, the extraction wound is sutured with simple interrupted sutures using 3-0 non absorbable black silk sutures. The rats were put supine position for 4 hours to maintain *HAp*-BTG stay in the post extraction slot. Evaluation days were 3, 7, 14, 21, and 28.

On the 3rd, 7th, 14th, 21st, and 28th days, conducting the termination each of 5 Wistar rats from each group, retracted, necropsied, decapitated, took a left mandibular bone fragment, and then immersed in 10% formalin solution for tissue fixation. After fixation, excised, and calcified, processed the left mandibular jaw for immersion in paraffin. Sections were made in a semi serial longitudinal manner with a thickness of 4 µm from the hemi mandibular containing the alveolar socket at 60 µm intervals and examined by hematoxylin-eosin (HE) staining and immunohistochemical (IHC) examination.

It used the HE staining to count the number of osteoblasts and osteoclasts. The slides were washed with PBS pH 7.4 three times for 5 minutes. Stained with hematoxylin-eosin for 10 minutes and soaked in tap water for 10 minutes. Then rinsed with dH2O and dehydrated with 30% and 50% alcohol for 5 minutes, respectively. Stained with eosin solution for 3 minutes, rinsed with 70%, 80%, 90%, and 95% alcohol twice, xylol three times, then mounted with permount adhesive and covered with a cover glass.[15]

The IHC staining examines BMP-2, OCN, OSX, and RUNX2 assay. The cut tissue preparation put on a glass object. The next steps were deparaffination, successively immersed into xylol three times (2 minutes each). Then immersed successively into ethanol with a gradually decreasing concentration starting from 100% ethanol (3 times of 1 minute), 95% (2 times of 1 minute), then 90%, 80%, and 70% each 1 minute. After that, washed with tap water for about 5 minutes, then added to 3% peroxide (30 minutes) to remove endogenous peroxidase. Then washed with water, rinsed with distilled water, then washed with PBS for 2 minutes each. Then put in 0.025% trypsin solution in PBS (pH 7.4) for 6 minutes at 37^{o} C. Then washed with PBS 3 times, 2 minutes each), and put into the primary antibody (mouse anti rat BMP monoclonal antibody) for 30 minutes, and washed with PBS (3 times, 2 minutes each), then put into the secondary antibodies, namely anti mouse biotinylated label for 30 minutes. Then washed with PBS (2 times, 2 minutes each), then put into streptavidin HRP label: 30 minutes, washed with PBS (3 times, 2 minutes each), put into chromogen substrate: 5 minutes (DAB solution), washed with PBS (3 times, 2 minutes each), then rinsed with distilled water, put into Mayer Hematoxylin for 6 minutes, washed with running water, and finally mounted and can be observed under a light microscope. Performed in the same way for OCN, OSX, and RUNX2, with the appropriate antibody.[1,15,21,22]

The examination of BMP-2, OSX, OCN, and RUNX2 expressions, osteoblast, and osteoclast, used a 1000x magnification light microscope in 20 microscopic fields. The mean results per microscopic field were tabulated and analyzed with SPSS version 25.

3. Results

3.1. The variable of cell count between treatment group versus control

The animal experiment was from January 18, 2020, to March 7, 2020. There were 50 Wistar rats of 2 major groups, 25 control and 25 treatment rats, spread into 3, 7, 14, 21, and 28 days sub group. All 50 rats were alive until the end of the experiment.

Six preparations of evaluated variables for each rat dental alveolar tissue were prepared and stained for each variable. The enumeration of cells per microscopic field, among 20 microscopic fields of 1000x magnification, is in (►**Table 1**). Evaluated variables are BMP-2, RUNX2, OSX, and OCN; OB and OC.

The results of examining the number of cells expressing BMP-2 between control and treatment group of observation days were in (►**Table 2**). In contrast, the different test results between observation days are in (Figure 1). The number of cells per microscopic field in the treatment group, days 3, 7, 14, 21, and 28, are higher and significantly different than the control (►**Table 2**). The increase in the number of cells in the treatment group occurred on days 3 to 7, 7 to 21, and then there was no significant change until day 28 (►**Figure 1**). It shows the peak of BMP-2 expression was on day 21. Whereas in the control group, the number of cells increased significantly on day 7, there were no significant changes until day 28. The results of BMP-2 expression are in (►**Figure 2**). Cells those express BMP-2 are marked in brown.

The results were similar for RUNX2 (►**Table 3**, ►**Figure 3**), OSX (►**Table 4**, ►**Figure 4**), and OCN (►**Table 5**, ►**Figure 5**). The number of cells expressing RUNX2 in the treatment group was higher and significantly different than the control group,

except on day 7. RUNX2 in the treatment group showed an increase in expression from day 3 to day 14, and there was no longer any significant difference until day 21 and 28.

The number of cells with OSX expression in the treatment group on days 3, 7, 14, 21, and 28 was higher and significantly different than the control group ($p<0.05$). Based on the evaluation days, there were no significant increase on day three compared to the control group, but it increased significantly on day 7. There was not a significant increase until days 14, 21 and 28. At the same time, in the control group, a significant rise in OSX-expressing cells occurred on day 28 compared to day 3.

There was a significant increase in OCN expression from day 3 to day 14, and then there was no significant increase until days 21 and 28 (►**Figure 5**).

The examination of osteoblasts (OB) with hematoxylin-eosin staining showed that since days 3, 7, 14, 21 and 28, the number of cells in the treatment group was significantly higher than the control group **(►Table 7**). While the number of cells between days of observation in the treatment group, the number of OB cells increased significantly on day 21, and then stable until 28 days (►**Figure 6**).

The results of the osteoclast (OC) had the opposite picture. Since days 3, 7, 14, 21, and 28, the number of cells in the treatment group was lower, and there was a significant difference compared to the control group ($p<0.05$) **(►Table 8**). While on evaluation days, a significant decrease occurred on day seven compared to day 3, then it remained constant, and there was no significant difference from day 7 to day 14, 21, and 28 (►**Figure 7**). The results of osteoclast cell expression are in (►**Figure 8**).

3.2. Tables and Figures

Table 1. The number of cells per light microscopic field with 1000 times magnification, on the histopathological examination (Immunohistochemistry for BMP-2, RUNX2, OSX, OCN and hematoxylin-eosin staining for OB, OC) of alveolar bone socket tissue.

Days	Variables	Cell number (mean) ± SD		p
		Control group	Treatment group	
D-3	BMP-2	6,41 ± 1,83	9,83 ± 0,68	0,011
	RUNX2	5,04 ± 0,54	9,71 ± 0,86	0,000
	OSX	6,83 ± 2,33	8,53 ± 2,48	0,530
	OCN	7,59 ± 1,92	11,86 ± 1,45	0,004
	OB	6,21 ± 4,40	15,06 ± 0,97	0,009
	OC	10,95 ± 1,90	8,40 ± 0,60	0,021
D-7	BMP-2	10,76 ± 1,97	15,19 ± 1,61	0,009
	RUNX2	8,38 ± 2,56	12,20 ± 2,71	0,051
	OSX	6,93 ± 2,47	13,64 ± 1,49	0,001
	OCN	11,14 ± 2,02	13,20 ± 1,87	0,133
	OB	7,47 ± 2,62	11,89 ± 2,26	0,021
	OC	12,85 ± 2,31	4,50 ± 2,16	0,009
D-14	BMP-2	11,58 ± 2,65	17,11 ± 1,98	0,006
	RUNX2	9,51 ± 1,60	16,68 ± 3,19	0,004
	OSX	8,63 ± 2,83	14,38 ± 1,37	0,004
	OCN	10,13 ± 3,77	17,51 ± 1,87	0,004
	OB	8,00 ± 2,09	13,85 ± 1,83	0,002
	OC	12,05 ± 2,11	4,50 ± 0,73	0,001
D-21	BMP-2	10,67 ± 1,20	18,44 ± 1,05	0,000
	RUNX2	10,41 ± 1,79	17,59 ± 2,07	0,000
	OSX	10,38 ± 3,76	15,87 ± 3,40	0,041
	OCN	12,33 ± 2,17	17,04 ± 1,70	0,005
	OB	12,36 ± 3,51	17,33 ± 2,29	0,029
	OC	12,57 ± 2,22	3,67 ± 0,69	0,000
D-28	BMP-2	13,41 ± 2,00	17,16 ± 1,44	0,009
	RUNX2	12,90 ± 1,83	16,54 ± 2,21	0,022
	OSX	12,60 ± 1,83	16,44 ± 2,52	0,025
	OCN	13,42 ± 1,26	15,99 ± 1,23	0,055
	OB	14,68 ± 3,24	18,34 ± 1,98	0,047

OC	15,18 ± 1,43	3,98 ± 0,73	0,000

BMP-2 (Bone morphogenic protein-2), RUNX2 (Runt-related transcription factor-2), OSX (Osterix), OCN (Osteocalcin), OB (Osteoblast), OC (Osteoclast)

Table 2. Description of mean, standard deviation, and difference test between groups of BMP-2 expression in the control and treatment groups on days 3, 7, 14, 21, and 28

Groups	Days of evaluation					P
	D-3	D-7	D-14	D-21	D-28	
Control	6,41±1,83	10,76±1,97	11,58±2,65	10,67±1,20	13,41±2,00	<0,001
Treatment	9,83±0,68	15,19±1,61	17,11±1,98	18,44±1,05	17,16±1,44	<0,01
P	<0,05	<0,01	<0,01	<0,001	<0,01	

Notes: D=days

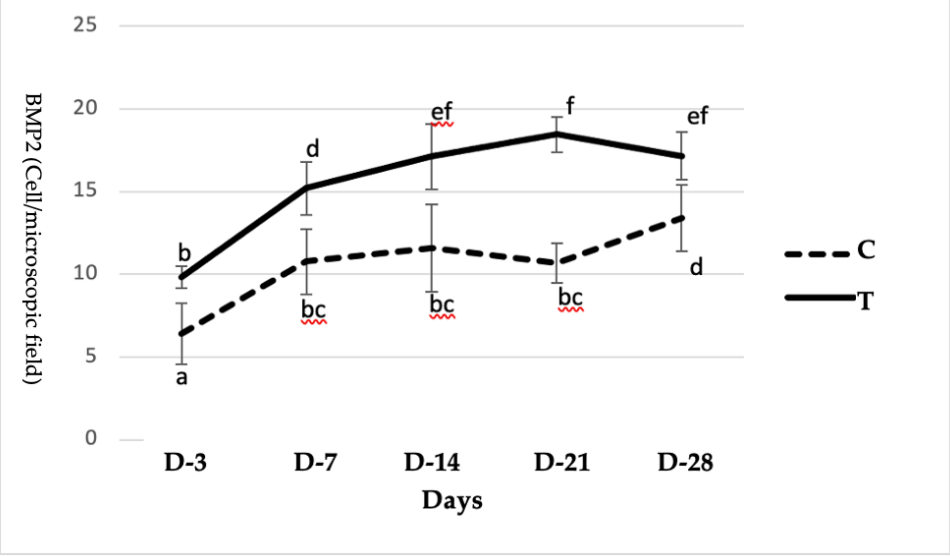

Figure 1. Comparison of mean of Bone morphogenic protein 2 (BMP-2) expression between study groups and time/day of observation.

Note: different letter notations (a, b, c, d, e, f) indicate significant differences between groups.

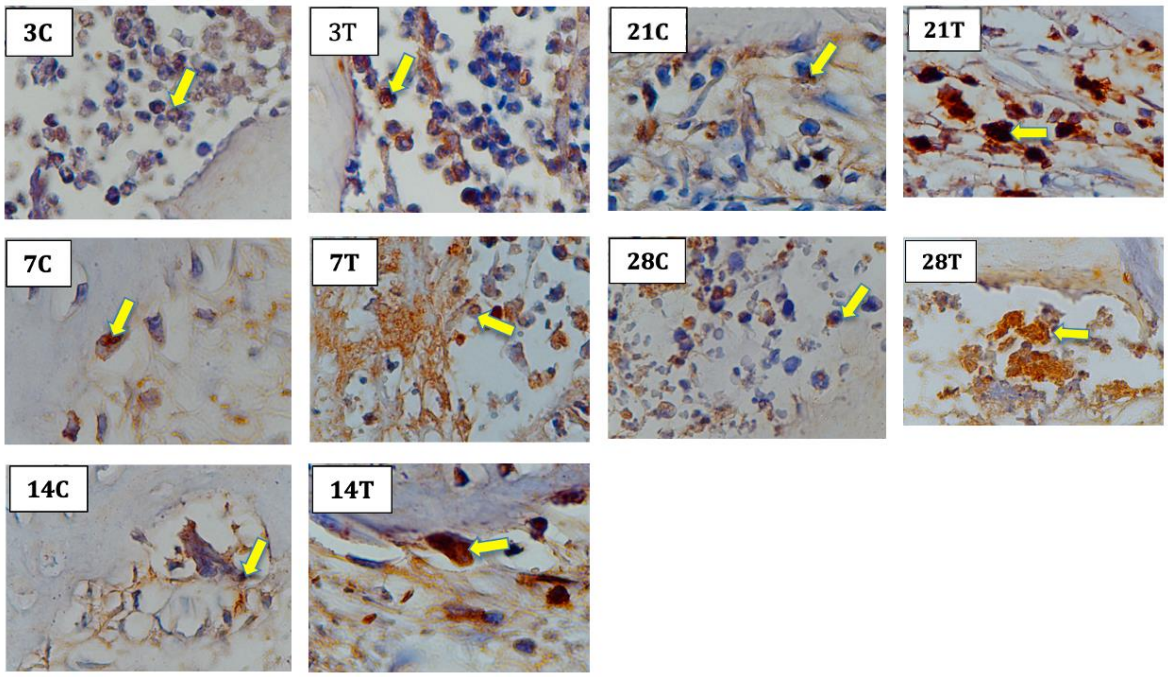

Figure 2. The results of immunohistochemistry (IHC) BMP-2 examination on the alveolar bone socket of the Wistar rat's teeth showed a picture of osteoblast cells with BMP-2 expression marked in brown.

Note: Numbers 3, 7, 14, 21, 28 = days of observation; C=control; T=Treatment; Arrows=brown are cells with BMP-2 expression.

Table 3. Description of mean, standard deviation, and difference test between RUNX-2 expression in the control and treatment groups, on days 3, 7, 14, 21 and 28

Group	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	5,04±0,54	8,38±2,56	9,51±1,60	10,41±1,79	12,90±1,83	<0,001
Treatment	9,71±0,86	12,20±2,71	16,68±3,19	17,59±2,07	16,54±2,21	<0,001
P	<0.001	>0.05	<0.01	<0.001	<0.05	

Notes: D=days

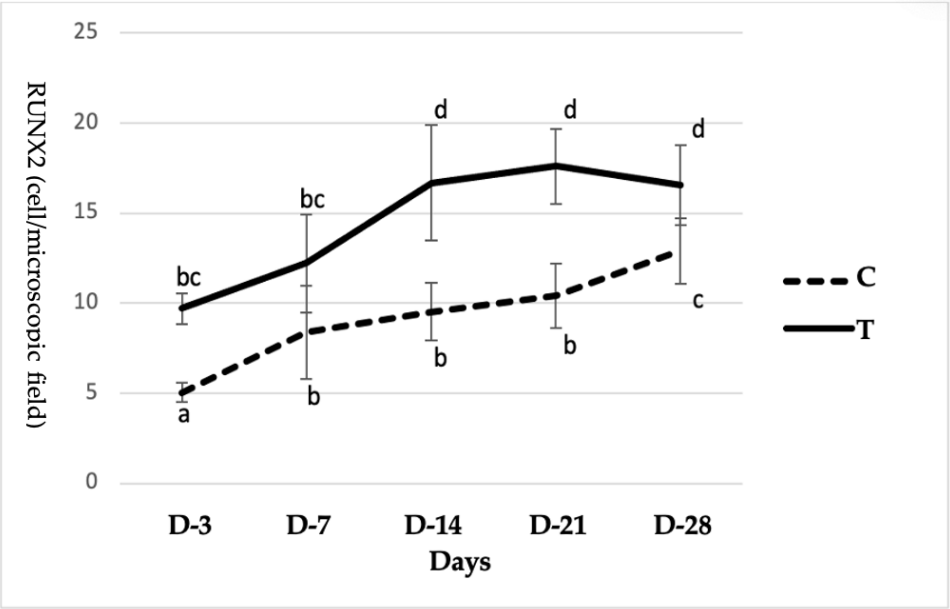

Figure 3. Comparison of mean of Runt-related transcription factor 2 (RUNX2) expression between study groups and time/day of observation.

Note: different letter notations (a, b, c, d, e, f) indicate significant differences between groups.

Table 4. Description of mean, standard deviation, and difference test between groups of OSX expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	6,83±2,33	6,93 ± 2,47	8,63±2,83	10,38±3,76	12,60±1,83	<0,05
Treatment	8,53±2,48	13,64±1,49	14,38±1,37	15,87±3,40	16,44±2,52	<0,01
P	>0,05	<0,01	<0,01	<0,05	<0,05	

Notes: D=days

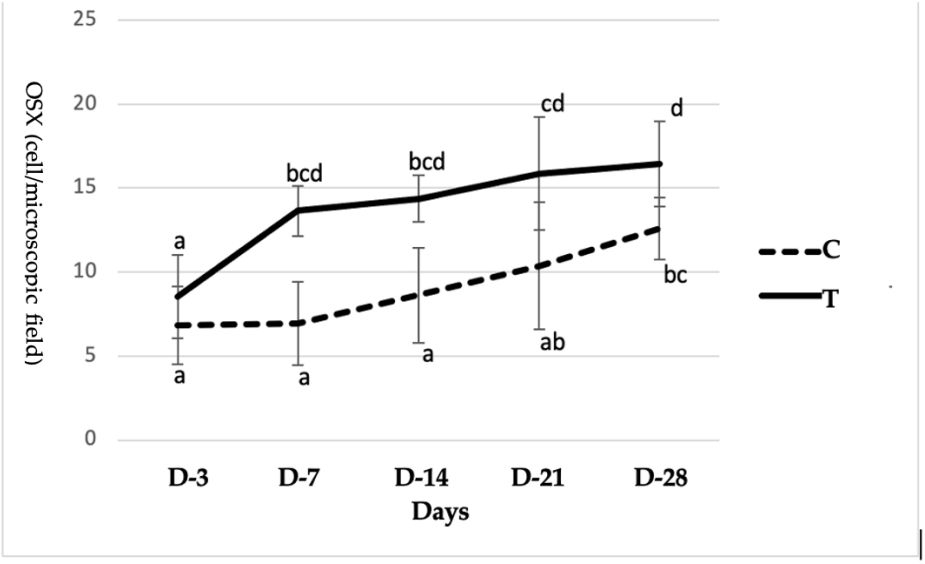

Figure 4. Comparison of mean Osterix (OSX) expression between study groups and time/day of observation.

Note: different notations (a,b,c,d) indicate differences between groups

Table 5. Description of the mean, standard deviation, and difference test between groups of OCN expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	7,59±1,92	11,14±2,02	10,13±3,77	12,23±2,17	13,42±1,26	<0,05
Treatment	11,86±1,45	13,20±1,87	17,51±1,87	17,04±1,70	15,99±1,23	<0,01
P	<0,01	>0,05	<0,01	<0,01	>0,05	

Notes: D=days

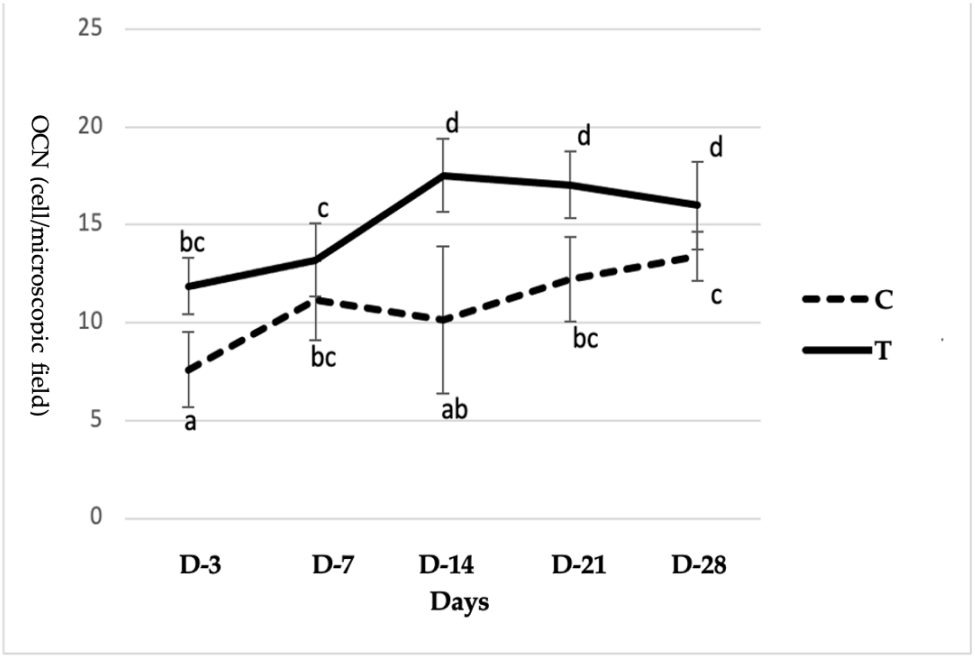

Figure 5. Comparison of mean Osteocalcin (OCN) expression between study groups and time/day of observation

Note: different notations (a,b,c,d) show differences between groups

Table 6. Description of mean, standard deviation, and difference test between groups of OB expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					p
	D-3	D-7	D-14	D-21	D-28	
Control	6,21±4,40	7,47±2,62	8,00±2,09	12,36±3,51	14,68±3,24	<0,01
Treatment	15,06±0,97	11,89±2,26	13,85±1,83	17,33±2,29	18,34±1,98	<0,01
P	<0,01	<0,05	<0,01	<0,05	<0,05	

Notes: D=days

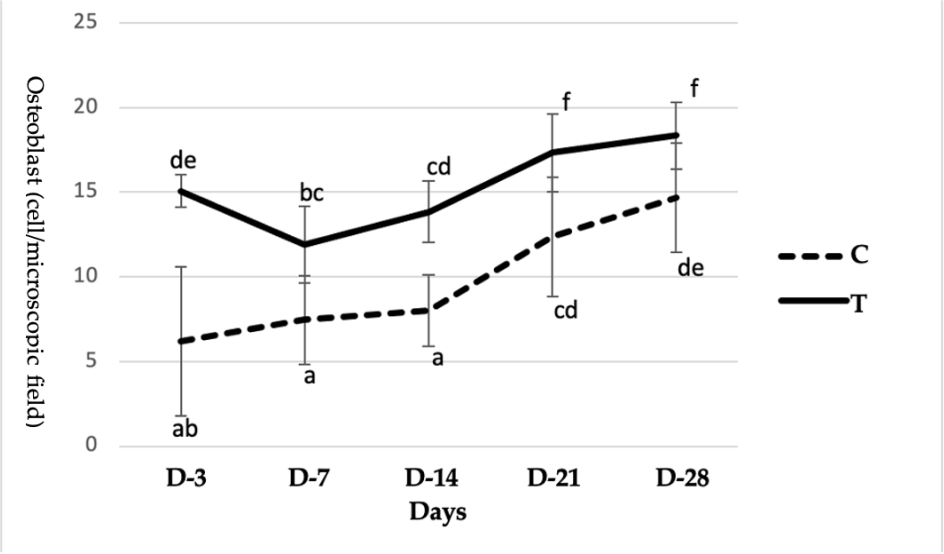

Figure 6. Comparison of the mean number of Osteoblast (OB) between study groups and time/day of observation.

Note: different notations (a,b,c,d,e,f) indicate differences between groups (Mann-Whitney test)

Table 7. Description of mean, standard deviation, and difference test between groups of OC expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	10,95±1,90	12,85±2,31	12,05±2,11	12,57±2,22	15,18±1,43	<0,05
Treatment	8,40±0,60	4,50±2,16	4,50±0,73	3,67±0,69	3,98±0,73	<0,01
P	<0,05	<0,01	<0,01	<0,001	<0,001	

Notes: D=days

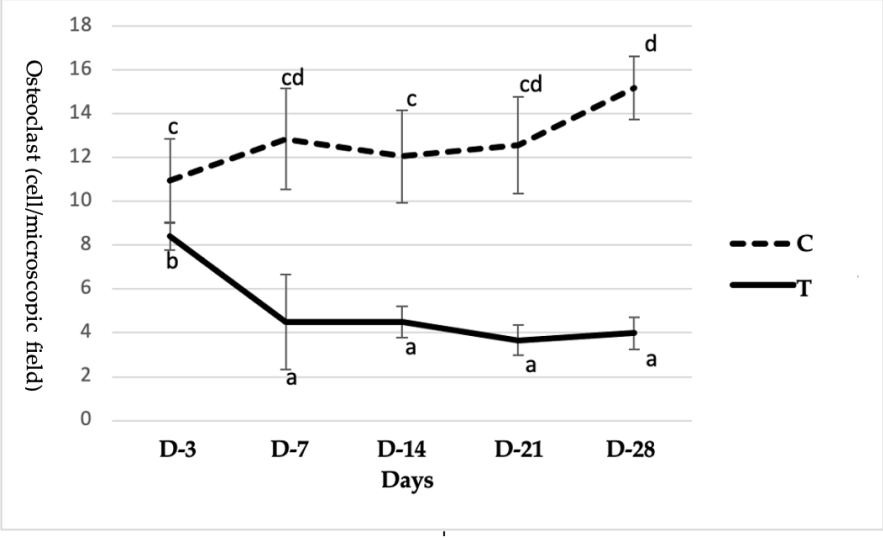

Figure 7. Comparison of mean Osteoclast (OC) expression between study groups and time/day of observation. Note: different notations (a,b,c,d) indicate differences between groups (Mann-Whitney test)

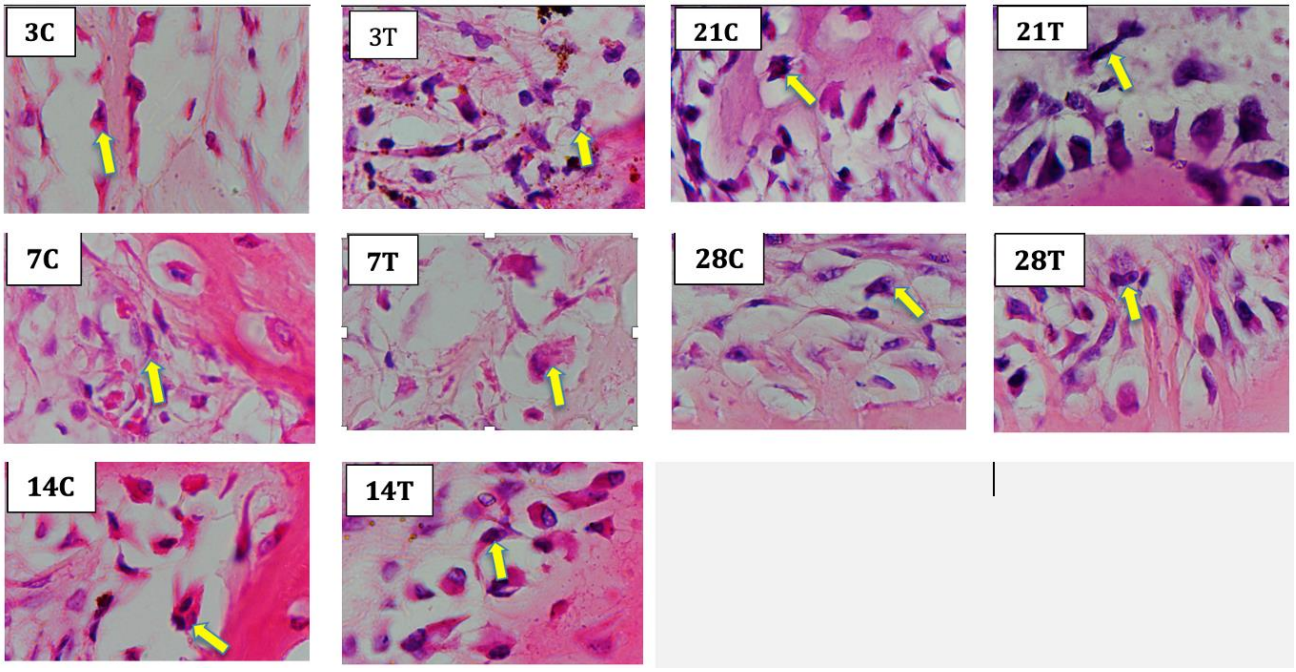

Figure 8. Osteoclast examination results in the alveolar bone socket of the *Norvegicus Wistar rat's* teeth, showing an image of osteoclast cells with multiple nuclear cells.

Note: Numbers 3, 7, 14, 21, 28 = days of observation; C=control; T=Treatment; Arrow=color are Osteoclast cells with multiple nuclei.

4. Discussion

HAp-BTG, as the test material in this study, is a particle resulting from calcination, sintering and milling process, with a size of about 3.5 microns. In various studies, the particle size of bone graft material is generally about 355 to 710 microns.[23] Particle sizes, both large (about 500 microns) and small in nano micron units, showed similar results. Studies with the same objective but using mesenchymal cells show an identical or similar impact on osteogenesis.[23] As an illustration, show at the peak of the expression of various indicators. OSX expression in this study, peak production could last until day 28, and OCN

until day 14. Wardhana et al showed that the expression of OCN and OPG were significant increase in day 7 & 14.[24]

In the treatment group, the increase in all osteogenesis indicators generally showed a significantly higher expression than the control group. It indicates the role of *HAp*-BTG in increasing the bone healing process in the area of the tooth alveolar socket.

It shows that peak expression in the treatment group was reached within 14 days (BMP-2, RUNX2, and OCN), day 7 (OSX). Meanwhile, the control group reached the peak on day 28 (BMP-2, RUNX2, OSX, OCN). The peak expression of the control group on day 28 (BMP-2, RUNX2, and OSX) was the same as that of the treatment group on day 7. It indicates that the *HAp*-BTG graft enhances and accelerates the process of osteogenesis. According to Thahir et al, the process of bone resorption and formation in male marmots takes about 2-4 weeks. This was due to the presence of osteoblasts, which perform actively to repair bone damage by forming collagen and non collagen proteoglycans and regulating the mineralization process between calcium and phosphate during the reversal phase.[25]

In the control group, OSX expression increased after day 3, but a significant increase was identified on day 28. In contrast, in the treatment group, a significant increase was identified since day 7 (►**Figure 4**) and continued to increase until day 28. Also, the expression of OCN in the control group increased on day 7, and then there was no significant increase until day 28 (►**Figure 5**); OB expression in the control group on day 28, there was no significant difference with the treatment group on day 21 (►**Figure 6**). From early to late healing stages, the mean of osteoblasts was consistently increasing.

Histology quantitative assessment showed that the mean number of osteoblasts was consistently higher in the DFDBBX group than in the DBBM group and control groups in 2 to 4 weeks.[15] This action is confirmed in a similar study by Kresnoadi et al, using mangosteen peel extract combined with demineralized freeze-dried bovine bone xenograft on osteocalcin, collagen 1, and osteoblast as alveolar bone regeneration in socket preservation also increase on days 7 and 30, the group treated with a combination of DFDBBX and mangosteen peel extract which had the highest expression and levels of osteocalcin, collagen 1, and osteoblasts.[26]

The number of OC in the control group there was no significant change between day 3 to day 7, day 7 to 14, day 14 to 21, and day 21 to 28. While in the treatment group, it decreased significantly on day 7, and then there were no significant changes from day 7 to day 14, day 21, and day 28. It showed that OC has a pivotal role in the finalization process of alveolar tissue growth.

The synthesis of hydroxyapatite has been widely used for bone repair, bone replacement, as a coating or filler for bones and teeth. However, it has been a lengthy application to develop hydroxyapatite as scaffolds.[27] *HAp* Scaffolds are *HAp* with a porous matrix where the size of the pores in hydroxyapatite scaffolds can vary, depending on the volume of a scaffold produced. It makes *HAp* scaffolds easier to implant into bone tissue, does not inhibit the growth of natural bone tissue, and can prevent displacement and loss of implants induced into the body. Hydroxyapatite scaffolds can serve as various materials, including polymers, ceramics, metals, and other composite materials.[28]

Hydroxyapatite is a source of calcium and phosphate, which is very important for the remineralization-demineralization of the enamel area.[29]

Bovine tooth graft has a role in osteogenesis in alveolar bone defects. After inserting the bone graft to the alveolar bone defect, there will be a blob of bone graft wrapped in blood in the early stages. Then on the 7th day, there will be an acute inflammatory response with an invasion of neutrophil cells, lymphocytes, and plasma cells. The inflammatory process that occurs causes the activation of pre-mesenchymal cells, growth factors, and inflammatory mediators that can cause pre-mesenchymal cells to differentiate into osteoblasts so that bone formation or osteogenesis will occur.[30]

BMP-2 is well known to be a strong inducer of bone formation and to play important roles in the development and regeneration of bone and cartilage.[31] The expression of BMP-2 in the mechanism of osteogenesis in various groups can be seen in ►**Table 3**, ►**Figures 1 and 2**, indicating significant differences between the treatment groups. The expression of BMP-2 in ►**Table 3** shows a significant difference in the expression of BMP-2 on days 3, 7, 14, 21, and 28 ($p<0.05$) between the treatment groups compared to the control group. The administration of *HAp*-BTG in the treatment group showed an increase in BMP-2, significantly higher than the control group ($p<0.001$). BMP signaling is one of the central signaling pathways that induce osteogenic differentiation and regulate bone formation. BMP induces osteogenesis through the role of autocrine, paracrine hormones, and the action of RUNX2.[32]

The osteoblastic differentiation and maturation events in the defect were evaluated by immunohistochemistry analysis which exhibits a significant increase in

expressions of RUNX2.[15] RUNX2 expression in various groups can be seen in ►**Table 4**, ►**Figure 3**, which shows significant differences between the treatment groups and the control groups. Table 4 shows significant differences in RUNX2 expression on days 3, 7, 14, 21, and 28 ($p<0.05$) between the control and treatment groups. However, it significantly increased RUNX2 expression on days 3, 7, and 14. After that, it increased again on day 21. Then there was no further increase until day 28 in the treatment group compared to the control group (►**Figure 3**).

RUNX2 was first detected in pre-osteoblasts and increased in the first week in immature osteoblasts. At the fourth week, RUNX2 expression decreased during the maturation process of osteoblasts, and RUNX2 expression was not significant in mature osteoblasts.[33] It is consistent with the results of this study, which showed that RUNX2 expression increased on day 14 and then was not any significant changes until day 28, and but were significant difference of RUNX2 on day 21 and 28 in the treatment group compared to the control group ($p<0.05$).

There was a significant difference between the treatment groups and the control group. OSX expression on day 3 showed no significant increase in treatment compared to control ($p>0.05$). In contrast, on days 7, 14, 21, and 28, there was a significant difference in OSX expression in the treatment group compared to the control group ($p<0,05$) (Figure 4). OSX is a novel transcription factor of Zinc Finger, an essential element for osteoblast differentiation and bone formation.[34,35] It plays an important role in the differentiation, maturation, or function of bone cells by regulating genes involved in different, and suggests a potential role in the bone microenvironment.[36]

Osteoblast expression in the mechanism of osteogenesis in this study showed the number of osteoblasts in various groups as in ►**Table 7** and ► **Figure 6**. On days 3, 7, 14, 21, and 28, the number of osteoblasts in the treatment group was significantly higher than in the control group. ($p<0.05$). However, based on time observations, there was a significant decrease in osteoblasts on day 7. On days 14 to 21, the number of osteoblasts increased and continued to increase significantly until day 28 **(►Figure 6**). Osteoblasts are responsible for collagen production (type I collagen) and non collagenous proteins. It includes osteocalcin (OCN), bone sialoprotein (BSP), osteopontin (OPN), and osteonectin. Osteoblasts also express some Alkaline Phosphatase (ALP) which helps mineralization.[37]

The number of osteoclasts in various groups is in Table 8 and Figure 7. On days 3, 7, 14, 21, and 28, the number of osteoclasts in the treatment group was significantly higher than in the control group ($p<0.05$) (►**Table 8**). However, based on time observations, there was a significant decrease in the number of osteoclasts on day 7, and then on days 14, 21 to 28, the number of osteoclasts remained low, the same as on day 7 (►**Figure 7**). Osteoclasts are the major cells responsible for bone resorption.[15] Osteoclasts play a role in the bone resorption process. Hydrogen ions formed from carbonic anhydrase enter the plasma membrane to dissolve the bone matrix during the resorption process. Different lysosomal enzymes, namely collagenase and cathepsin K, are released to digest the bone matrix.[38] The low number of osteoclasts indicates that bone growth continues throughout this experiment, which is indicated by high osteoblasts and low osteoclasts.

There is something new from this study: the mechanism of alveolar bone osteogenesis after administration of *HAp*-BTG on day three until 28 through the BMP-2, OSX, OCN, and RUNX2 number osteoblasts and osteoclasts in vivo. The *HAp*-BTG scaffold material was proven to have the ability to induce osteogenesis in the alveolar bone socket of the *Rattus norvegicus strain Wistar rats* in vivo. This *HAp*-BTG scaffold has potential as a mineral because it secretes active metabolites such as cytokines and growth factors. These results show the pathway of the influence of *HAp*-BTG and BMP2 expression. The expression of BMP-2 affects the expression of RUNX2, which is the beginning of the process of osteogenesis. RUNX2 plays a role in the differentiation of MSCs into osteoprogenitors. The RUNX2 pathway affects the expression of OSX, which is the final stage of the osteogenic process. The role of OSX is to induce the differentiation of osteoprogenitors into preosteoblasts. The OSX pathway affects OCN expression, which indicates that preosteoblasts differentiate into osteoblasts. Osteocalcin is the most abundant protein matrix found in bone. Osteoblasts express osteocalcin in the bone matrix during alveolar bone remodeling.[25] Alveolar bone osteogenesis will be supported by the expression of OCN and the number of osteoblasts and a decrease in the number of osteoclasts in vivo.

In osteoblastic differentiation studies, Runx2 is one of the most common markers investigated. It showed that hydroxyapatite induces the differentiation of osteoblasts by upregulating Runx2. [39,40,41] Hydroxyapatite also induces OSX expression, which causes osteoblastic differentiation of osteoblast progenitor cells.[42] OSX involve in an early stage of osteoblast differentiation. The overexpression of OSX can inhibit the late stage of

osteoblast differentiation.[43] OSX plays a role in bone homeostasis. Inactivation of OSX in the postnatal period caused defects in osteoblast function, followed by decreased bone formation.[44] OSX and RUNX2 regulated the unique cartilage matrix-associated protein. OCN is known as a gamma-carboxyglutamate protein expressed by osteoblasts. Hydroxyapatite also induces osteoblast differentiation which shows an increase in OCN. [40,41,45,46]

Hydroxyapatite plays a role in the BMP signaling pathway inducing osteogenic differentiation. This mechanism proceeds via canonical signals, as reported by Khotib et al. [47] BMP-2 act to significantly enhance osteogenesis and angiogenic differentiation.[48] BMP-2 may stimulate extramedullary bone regeneration.[31] Nam et al. reported that BMP-2 combined with both hydroxyapatite and bovine-derived xenografts. It showed effectively enhances the alveolar ridge in the treatment of augmentation of the alveolar ridge, and BMP-2 in combination with hydroxyapatite is especially effective in repairing complex bone defects.[49]

5. Conclusions

(1) Administration of *HAp*-BTG into the alveolar bone socket of Wistar rats can increase the expression of BMP-2, RUNX2, OSX, OCN, the number of osteoblasts and decrease the number of osteoclasts;

(2) The bone healing process of alveolar bone socket post dental extraction in Wistar rats is higher and faster after induction with *HAp*-BTG.

Author Contributions: For research articles with several authors, since developing the protocol until finalizing the manuscript. The following statements should be put "Conceptualization, N.Z., S.K., E.M.S.; Data analysis, N.Z.; Data collection, N.Z.; Writing draft preparation, N.Z., S.K., E.M.S.; Draft editing and references, N.Z., S.K., E.M.S., D.D.P., M.M.S.N.M.; Final writing and editing, N.Z., S.K., E.M.S., D.D.P., M.M.S.N.M.; Funding application, N.Z.; All authors agreed to the published final version.

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Informed Consent Statement: Not applicable

Data Availability Statement: The data of this study are available from the corresponding author on request.

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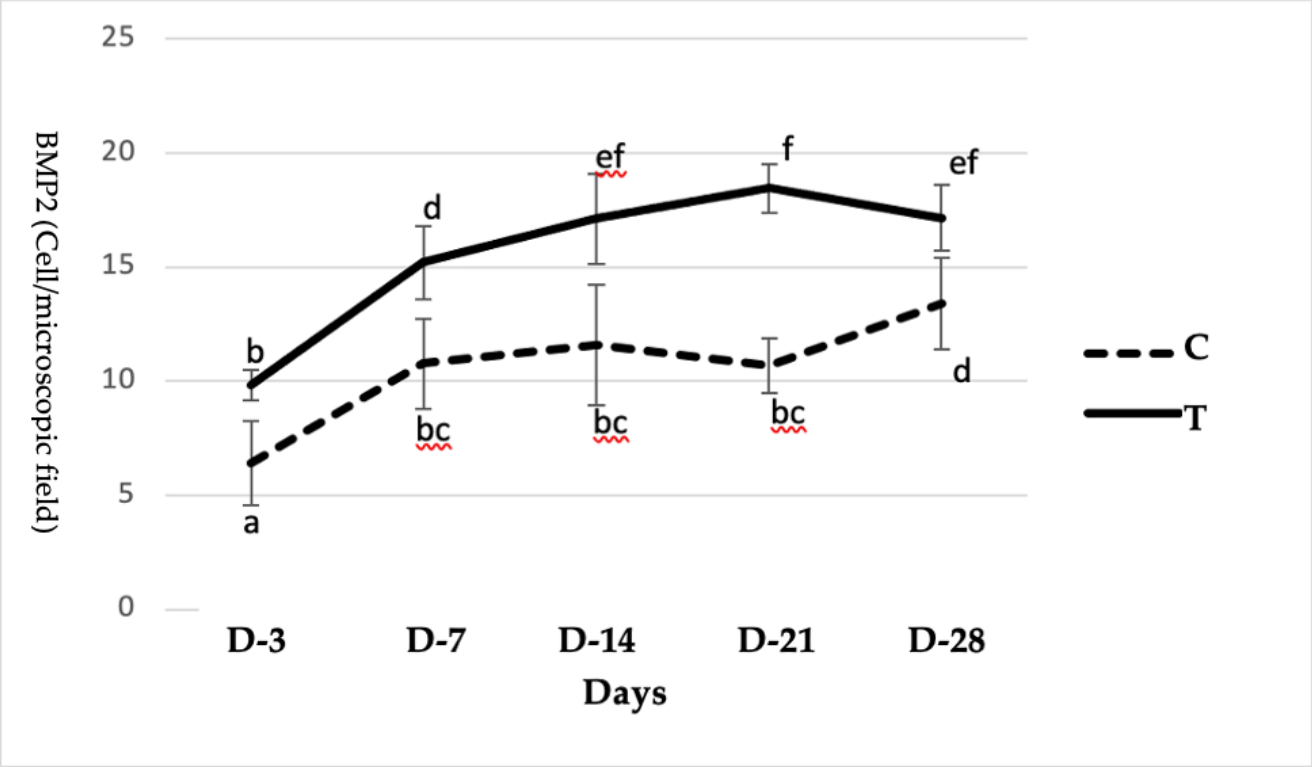

Figure 1. Comparison of mean of Bone morphogenic protein 2 (BMP-2) expression between study groups and time/day of observation.

Note: different letter notations (a, b, c, d, e, f) indicate significant differences between groups

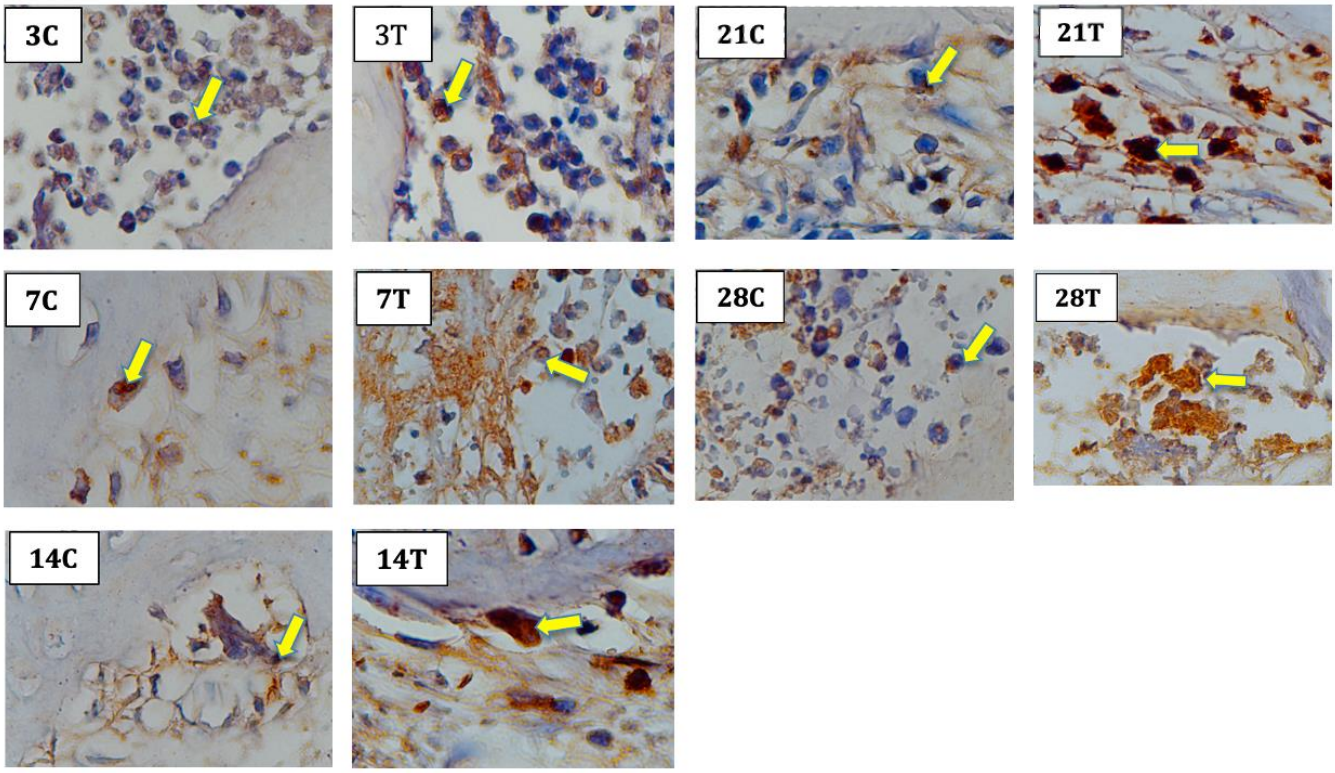

Figure 2. The results of immunohistochemistry (IHC) BMP-2 examination on the alveolar bone socket of the Wistar rat's teeth showed a picture of osteoblast cells with BMP-2 expression marked in brown.

Note: Numbers 3, 7, 14, 21, 28 = days of observation; C=control; T=Treatment; Arrows=brown are cells with BMP-2 expression.

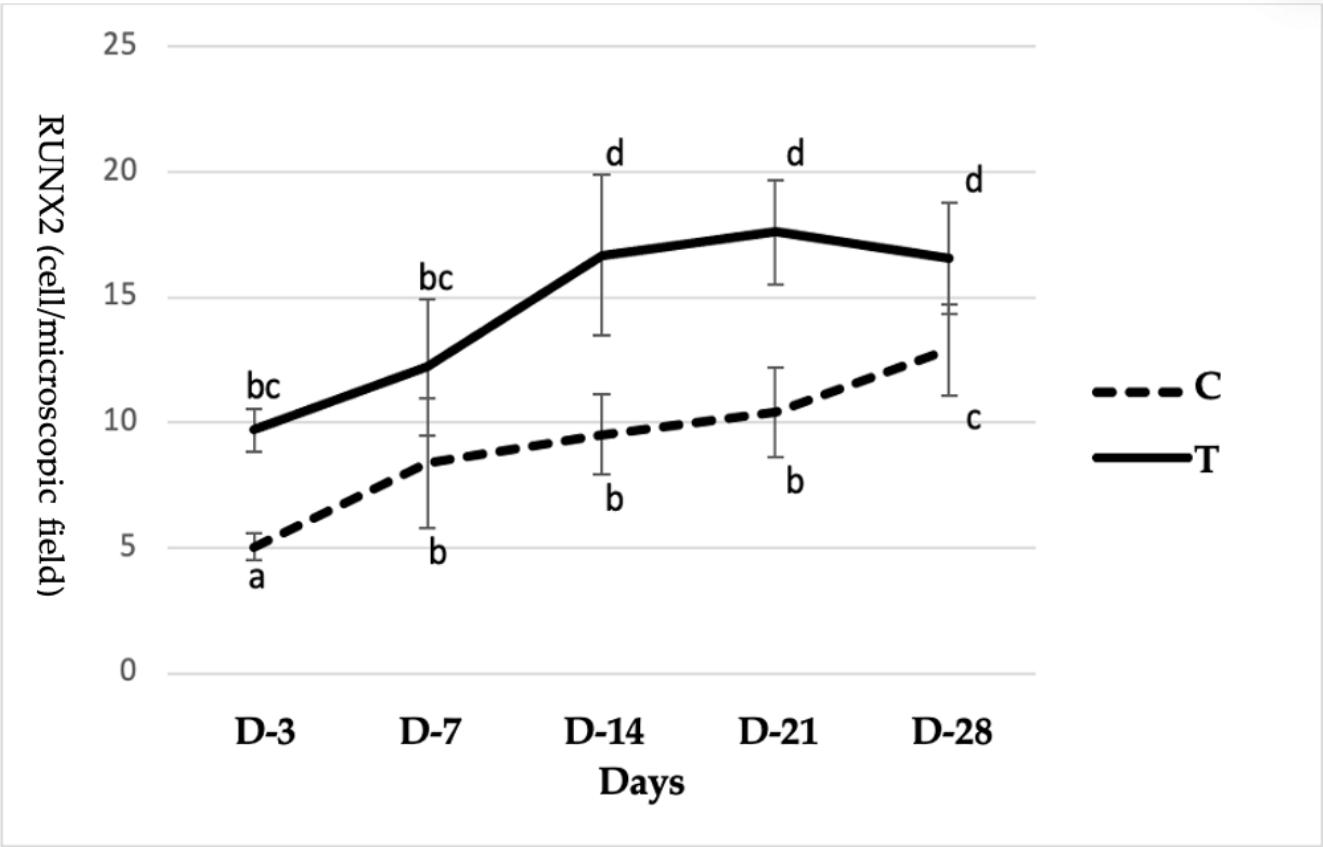

Figure 3. Comparison of mean of Runt-related transcription factor 2 (RUNX2) expression between study groups and time/day of observation.

Note: different letter notations (a, b, c, d, e, f) indicate significant differences between groups

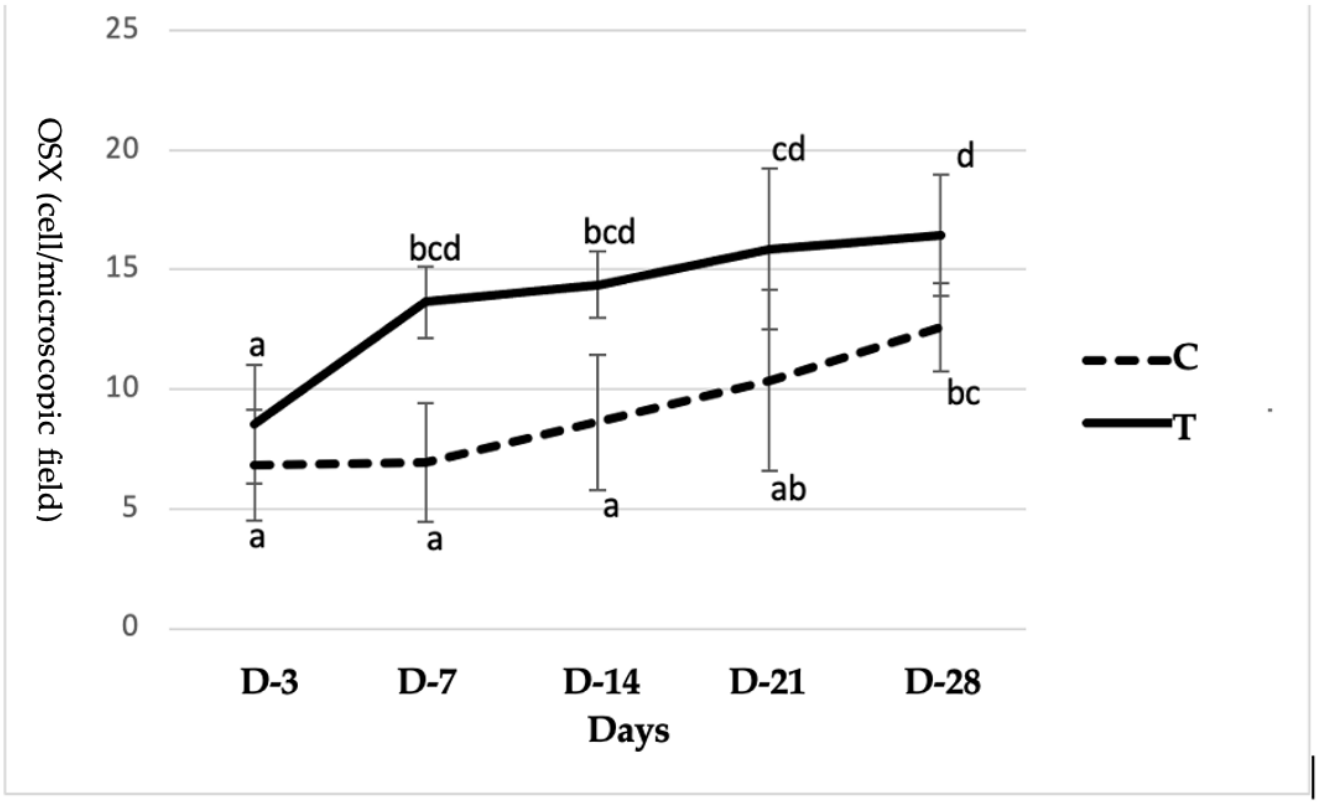

Figure 4. Comparison of mean Osterix (OSX) expression between study groups and time/day of observation.

Note: different notations (a,b,c,d) indicate differences between groups

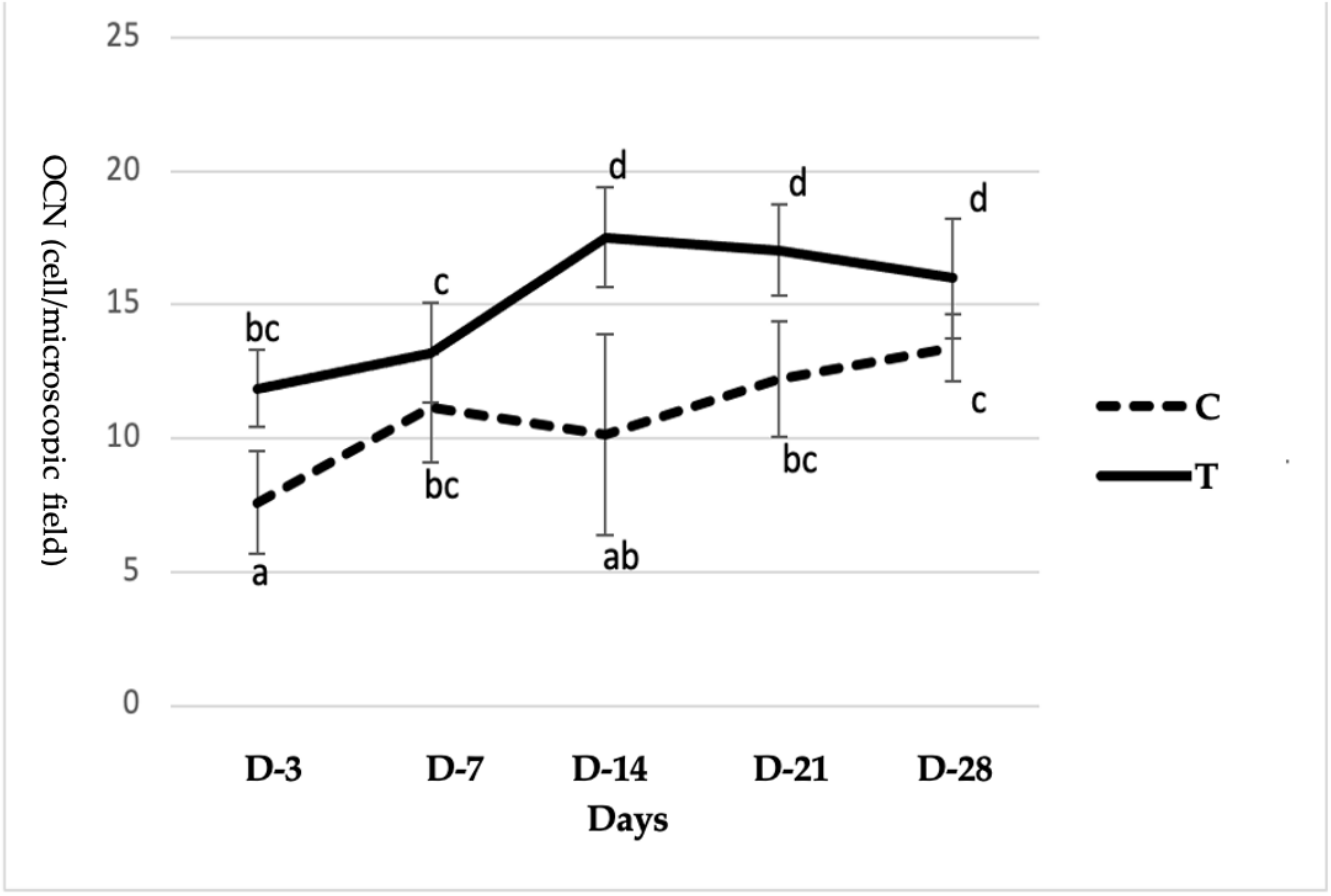

Figure 5. Comparison of mean Osteocalcin (OCN) expression between study groups and time/day of observation

Note: different notations (a,b,c,d) show differences between groups

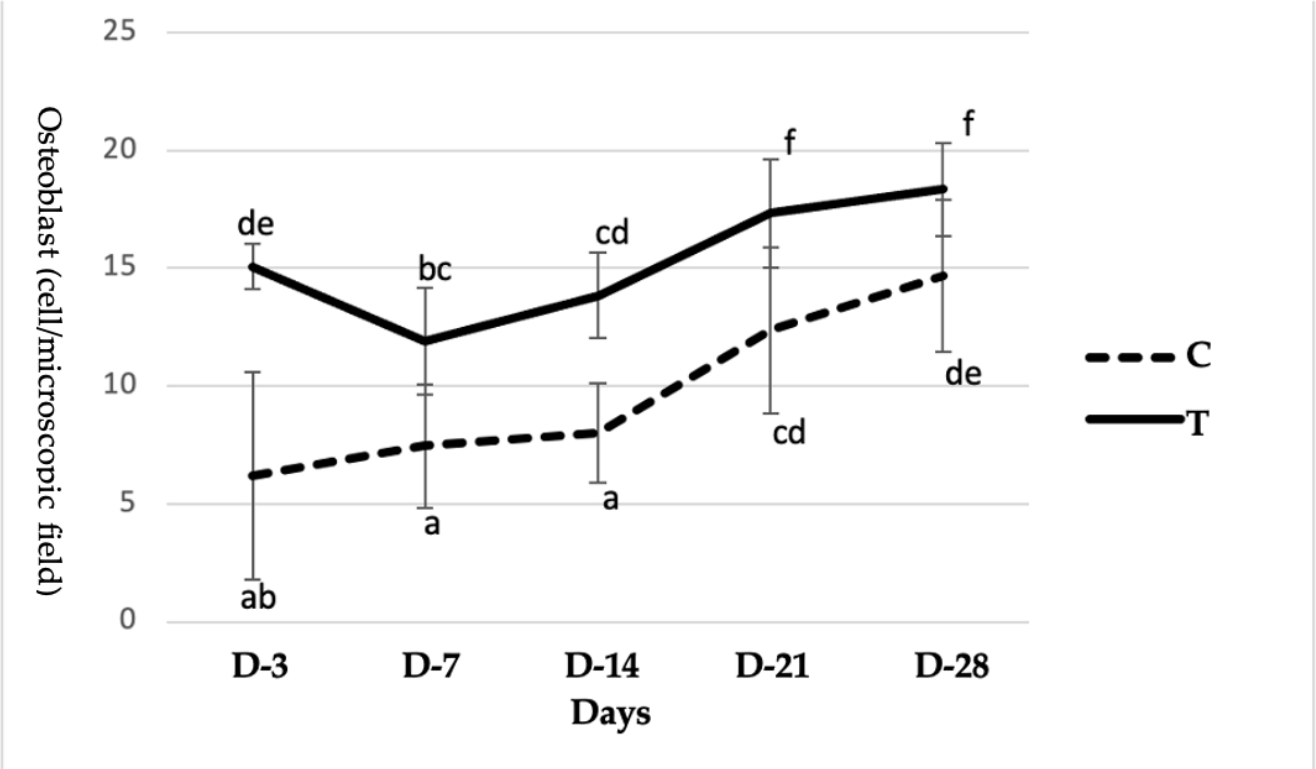

Figure 6. Comparison of the mean number of Osteoblast (OB) between study groups and time/day of observation.

Note: different notations (a,b,c,d,e,f) indicate differences between groups (Mann-Whitney test)

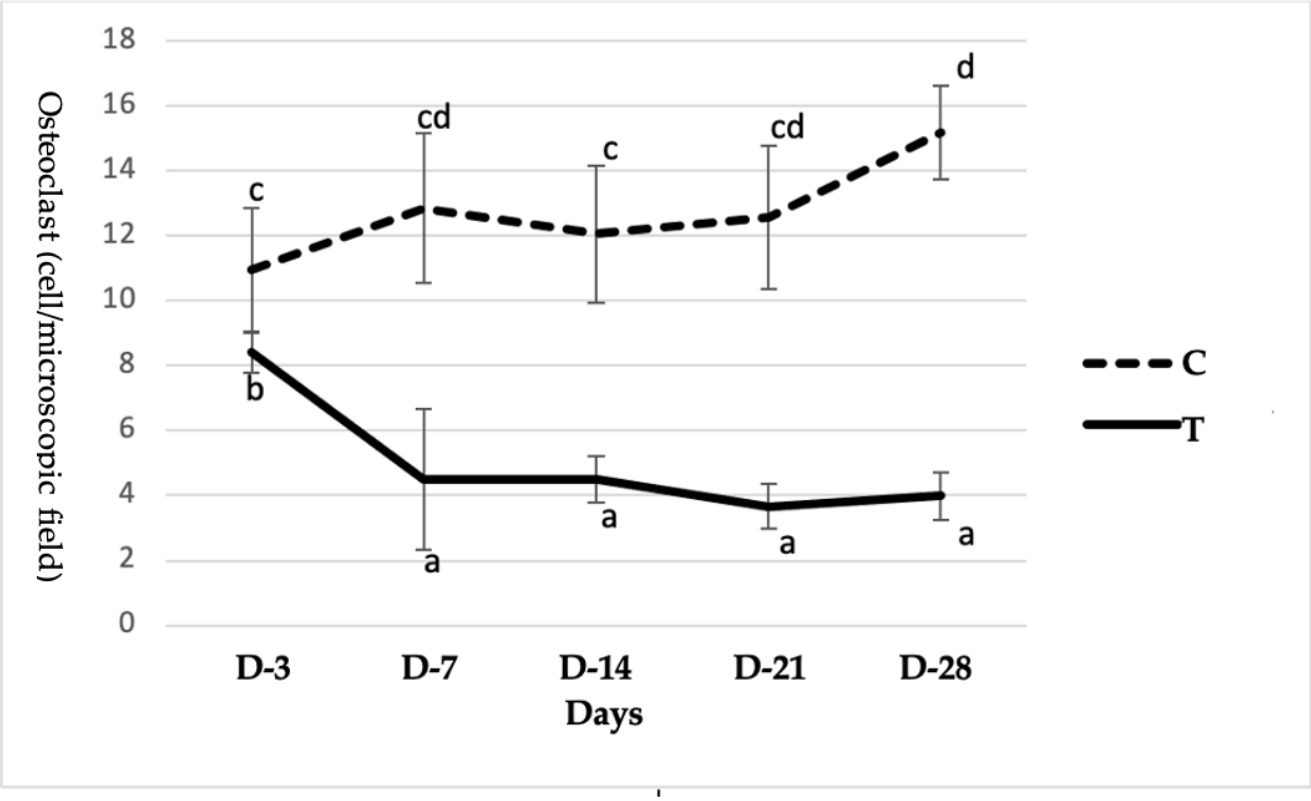

Figure 7. Comparison of mean Osteoclast (OC) expression between study groups and time/day of observation. Note: different notations (a,b,c,d) indicate differences between groups (Mann-Whitney test)

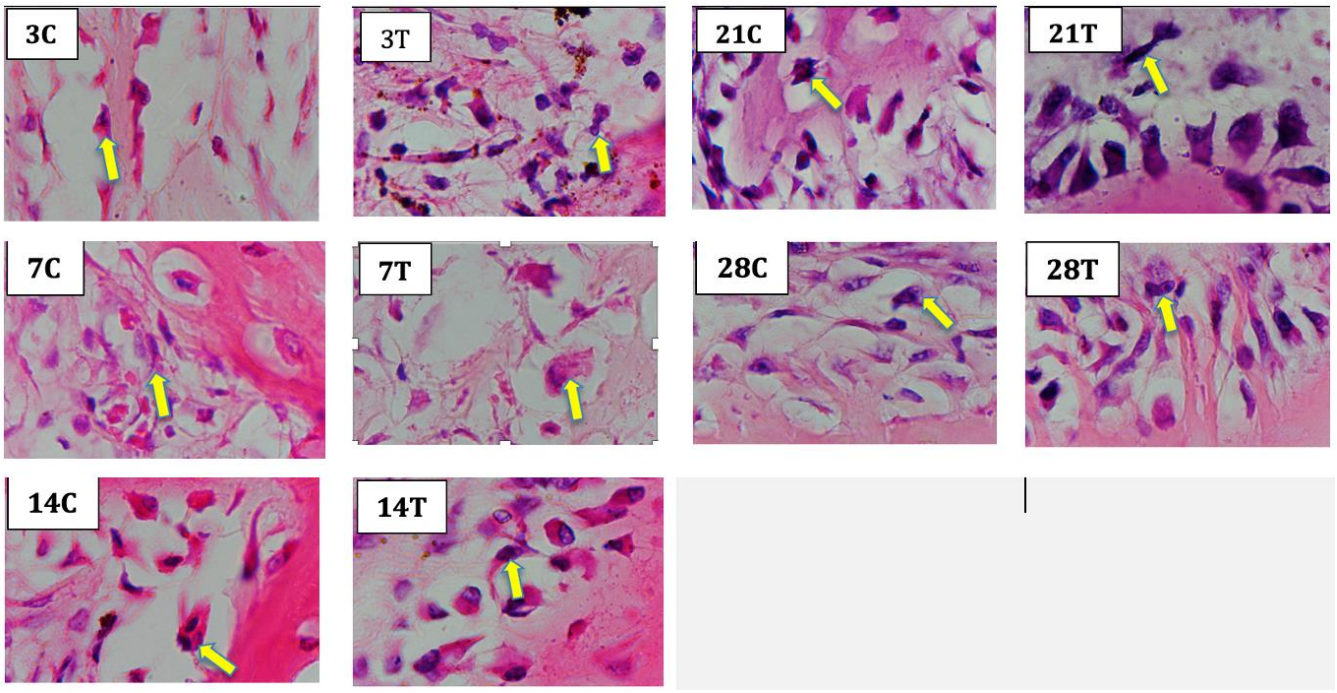

Figure 8. Osteoclast examination results in the alveolar bone socket of the *Norvegicus Wistar rat's* teeth, showing an image of osteoclast cells with multiple nuclear cells.

Note: Numbers 3, 7, 14, 21, 28 = days of observation; C=control; T=Treatment; Arrow=color are Osteoclast cells with multiple nuclei.

3.2. *Tables and Figures*

Table 1. The number of cells per light microscopic field with 1000 times magnification, on the histopathological examination (Immunohistochemistry for BMP-2, RUNX2, OSX, OCN and hematoxylin-eosin staining for OB, OC) of alveolar bone socket tissue.

Days	Variables	Cell number (mean) ± SD		P
		Control group	Treatment group	
D-3	BMP-2	6,41 ± 1,83	9,83 ± 0,68	0,011
	RUNX2	5,04 ± 0,54	9,71 ± 0,86	0,000
	OSX	6,83 ± 2,33	8,53 ± 2,48	0,530
	OCN	7,59 ± 1,92	11,86 ± 1,45	0,004
	OB	6,21 ± 4,40	15,06 ± 0,97	0,009
	OC	10,95 ± 1,90	8,40 ± 0,60	0,021
D-7	BMP-2	10,76 ± 1,97	15,19 ± 1,61	0,009
	RUNX2	8,38 ± 2,56	12,20 ± 2,71	0,051
	OSX	6,93 ± 2,47	13,64 ± 1,49	0,001
	OCN	11,14 ± 2,02	13,20 ± 1,87	0,133
	OB	7,47 ± 2,62	11,89 ± 2,26	0,021
	OC	12,85 ± 2,31	4,50 ± 2,16	0,009
D-14	BMP-2	11,58 ± 2,65	17,11 ± 1,98	0,006
	RUNX2	9,51 ± 1,60	16,68 ± 3,19	0,004
	OSX	8,63 ± 2,83	14,38 ± 1,37	0,004
	OCN	10,13 ± 3,77	17,51 ± 1,87	0,004
	OB	8,00 ± 2,09	13,85 ± 1,83	0,002
	OC	12,05 ± 2,11	4,50 ± 0,73	0,001
D-21	BMP-2	10,67 ± 1,20	18,44 ± 1,05	0,000
	RUNX2	10,41 ± 1,79	17,59 ± 2,07	0,000
	OSX	10,38 ± 3,76	15,87 ± 3,40	0,041
	OCN	12,33 ± 2,17	17,04 ± 1,70	0,005
	OB	12,36 ± 3,51	17,33 ± 2,29	0,029
	OC	12,57 ± 2,22	3,67 ± 0,69	0,000
D-28	BMP-2	13,41 ± 2,00	17,16 ± 1,44	0,009
	RUNX2	12,90 ± 1,83	16,54 ± 2,21	0,022
	OSX	12,60 ± 1,83	16,44 ± 2,52	0,025
	OCN	13,42 ± 1,26	15,99 ± 1,23	0,055
	OB	14,68 ± 3,24	18,34 ± 1,98	0,047
	OC	15,18 ± 1,43	3,98 ± 0,73	0,000

BMP-2 (Bone morphogenic protein-2), RUNX2 (Runt-related transcription factor-2), OSX (Osterix), OCN (Osteocalcin), OB (Osteoblast), OC (Osteoclast)

Table 2. Description of mean, standard deviation, and difference test between groups of BMP-2 expression in the control and treatment groups on days 3, 7, 14, 21, and 28

Groups	Days of evaluation					P
	D-3	D-7	D-14	D-21	D-28	
Control	6,41±1,83	10,76±1,97	11,58±2,65	10,67±1,20	13,41±2,00	<0,001
Treatment	9,83±0,68	15,19±1,61	17,11±1,98	18,44±1,05	17,16±1,44	<0,01
P	<0,05	<0,01	<0,01	<0,001	<0,01	

Notes: D=days

Table 3. Description of mean, standard deviation, and difference test between RUNX-2 expression in the control and treatment groups, on days 3, 7, 14, 21 and 28

Group	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	5,04±0,54	8,38±2,56	9,51±1,60	10,41±1,79	12,90±1,83	<0,001
Treatment	9,71±0,86	12,20±2,71	16,68±3,19	17,59±2,07	16,54±2,21	<0,001
P	<0.001	>0.05	<0.01	<0.001	<0.05	

Notes: D=days

Table 4. Description of mean, standard deviation, and difference test between groups of OSX expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	6,83±2,33	6,93 ± 2,47	8,63±2,83	10,38±3,76	12,60±1,83	<0,05
Treatment	8,53±2,48	13,64±1,49	14,38±1,37	15,87±3,40	16,44±2,52	<0,01
P	>0,05	<0,01	<0,01	<0,05	<0,05	

Notes: D=days

Table 5. Description of the mean, standard deviation, and difference test between groups of OCN expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	7,59±1,92	11,14±2,02	10,13±3,77	12,23±2,17	13,42±1,26	<0,05
Treatment	11,86±1,45	13,20±1,87	17,51±1,87	17,04±1,70	15,99±1,23	<0,01
P	<0,01	>0,05	<0,01	<0,01	>0,05	

Notes: D=days

Table 6. Description of mean, standard deviation, and difference test between groups of OB expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					p
	D-3	D-7	D-14	D-21	D-28	
Control	6,21±4,40	7,47±2,62	8,00±2,09	12,36±3,51	14,68±3,24	<0,01
Treatment	15,06±0,97	11,89±2,26	13,85±1,83	17,33±2,29	18,34±1,98	<0,01
P	<0,01	<0,05	<0,01	<0,05	<0,05	

Notes: D=days

Table 7. Description of mean, standard deviation, and difference test between groups of OC expression in the control and treatment groups on days 3, 7, 14, 21 and 28

Groups	Evaluation days					P
	D-3	D-7	D-14	D-21	D-28	
Control	10,95±1,90	12,85±2,31	12,05±2,11	12,57±2,22	15,18±1,43	<0,05
Treatment	8,40±0,60	4,50±2,16	4,50±0,73	3,67±0,69	3,98±0,73	<0,01
P	<0,05	<0,01	<0,01	<0,001	<0,001	

Notes: D=days