World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

Check for updates

Application of chitosan-gelatin-carbonate hydroxy apatite scaffold toward the number of osteoblasts in alveolar bone defects in Wistar Rats

Devi Rianti [1,*], Noor Zain Salsabilla Prabowo [2], Maryama Aflaha [2], Titien Hary Agustantina [1] and Adriansyah syahrom [3]

[1] *Department of Dental Materials, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.*
[2] *Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.*
[3] *Medical Devices and Technology Centre (MEDITEC), Institute of Human Centered and Engineering (iHumEn), University Teknologi, Malaysia, 81310 UTM Skudai, Johor, Malaysia.*

World Journal of Advanced Research and Reviews, 2023, 18(03), 768–775

Publication history: Received on 29 March 2023; revised on 09 June 2023; accepted on 12 June 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.18.3.0840

Abstract

Tooth extraction may cause bone defects that require regenerative therapy. Chitosan, gelatin, and carbonate hydroxyapatite can help increase osteoblast proliferation and differentiation, which plays a role in the bone regeneration process. Scaffold made from chitosan-gelatin carbonate hydroxyapatite has promising characteristics that may help enhance the bone regeneration process. This study aimed to determine the effect of chitosan-gelatin carbonate hydroxyapatite (CG:CHA) scaffold application towards the number of osteoblasts on alveolar bone defect in Wistar rats after tooth extraction. *In vivo* experimental laboratory research conducted with post-test-only control group design. The Wistar rats were divided into 6 groups, tooth extraction was conducted, and in the treatment group the C-G:CHA scaffold was applied on the sockets afterward. After 7, 14, and 21 days of scaffold application, jaw resection was done to observe the number of osteoblasts by HE histological examination. The result data were analyzed using a one-way ANOVA test. Data analysis using one-way ANOVA test results in P=0 (P <0,05) which indicates a significant difference between the control and treatment groups. The chitosan-gelatin carbonate hydroxyapatite (C-G:CHA) scaffold can increase the number of osteoblasts.

Keywords: Scaffold; Chitosan; Gelatin; Carbonate hydroxyapatite; Osteoblast; Medicine

1. Introduction

Tissues that have been damaged by inflammation, neoplastic, or trauma in the dental, oral, and maxillofacial areas require regenerative therapy[1]. Bone reconstruction measures can be performed to restore bone volume and density to restore bone function[2].

Tooth extraction can cause changes in the alveolar ridge[3]. After tooth extraction, the alveolar ridge undergoes a process of bone healing which consists of an inflammatory phase, to a remodeling phase, which in some cases is accompanied by bone defects in the form of resorption of crestal bone which results in changes in the dimensions of the ridge[4,5,6].

The process of bone healing is a complex biological and biomechanical process[7]. The formation of osteoblasts, mineralization of the extracellular matrix and osteoid, and the formation of osteocytes are several processes that play an important role in bone healing [8]. Osteoblasts have a role in bone growth and remodeling. Osteoblasts play a role in the synthesis, deposition, and mineralization of bone matrix by producing osteoid, secreting, and storing bone matrix proteins[9,10].

* Corresponding author: Devi Rianti

Several studies have utilized a tissue engineering-based approach to develop a biological substitution that can help construct, maintain, repair, and restore the function of bone tissue that has been damaged. One of the main components in tissue engineering is the scaffold[11]. Scaffold plays a role in facilitating the growth of mesenchymal stromal cells (MSC), supporting tissue growth and development which acts as an extracellular matrix, cell adhesion for proliferation, and differentiation to form new tissues. Criteria for an ideal scaffold are a scaffold with biocompatible, biodegradable, osteoconductive, osteoinductive properties, capable of supporting pressure loads, having a porous structure with a size of >100μm so that it can help cell penetration, and has a high surface area/volume ratio so it can support attachment, proliferation, and cell differentiation[2,12,13]. There are three types of materials that are generally used as scaffold base materials, namely natural polymers, such as chitosan, synthetic polymers, and bioceramics[14].

The chitosan-gelatin carbonate hydroxyapatite (C-G:CHA) scaffold can be made with a ratio of 30:70 (w/w) to approximate the weight ratio of the original bone. It is estimated that the mineral or inorganic component of bone contains about 60 to 70% of the total bone weight, while the rest are organic components such as protein and collagen[15]. According to research conducted by Yuliati et al, chitosan-gelatin hydroxyapatite (C-G:CHA) scaffold with a ratio of 30:70 (w/w) is a good biomaterial because it has the appropriate porosity as a medium for osteoblast growth[16].

In vitro and *in vivo* studies are important before trials on humans, to ensure the safety and effectiveness of the materials being developed[17]. Research by Dewi & Triawan showed that the application of an apatite-chitosan carbonate scaffold as a bone substitute in rat tibia bone defects can increase the number of osteoblasts on days 7th, 14th, and 21st after application[15]. Based on a study by Ariani, it was found that the results of a significant increase in mouse osteoblast-like cell (MC3T3-E1) proliferation were observed 7, 14, and 21 days after the application of chitosan apatite carbonate scaffold compared to the control group[18].

In a study conducted by Sularsih, it was stated that the application of chitosan to the socket after tooth extraction in Wistar rats could increase the number of osteoblasts, fibroblasts, and type I collagen on days 7 and 14[19]. In the process of bone healing, days 7 to 9 are thought to be the peak of young callus formation. On the 14th day, the peak of the hard callus formation process occurred, and the remodeling process began to occur around the third week or the 21st day[20]. Based on the above background, this study was conducted to analyze the application of the chitosan-gelatin-carbonate hydroxyapatite (C-G:CHA) scaffold with a ratio of 30:70 (w/w) can affect the number of osteoblasts in alveolar bone defects in Wistar rats (*Rattus norvegicus*) 7, 14, and 21 days after tooth extraction.

2. Material and methods

This research is an *in vivo* laboratory experimental study with a post-test only control group design. Samples of male Wistar rats (*Rattus norvegicus*) aged 12-16 weeks and weighing 200–250 g totaling 24 rats were divided into six treatment and control groups with a total sample of 4 individuals per group. This research met ethical requirements and was approved by the UNAIR Faculty of Dentistry Ethics Committee No. 560/HRECC.FODM/VIII/2022.

The tooth extraction procedure in Wistar rats was carried out using xylazine anesthesia and intramuscular injection of ketamine as much as 0.1 ml/10 g body weight in experimental animals of Wistar rats. The rat tooth extraction area was performed asepsis using Povidone Iodine 10%, then the mandibular incisors were extracted using tooth extraction pliers and periotome to make bone defects in the sockets of the experimental animals. Next, a C-G:CHA scaffold with a ratio of 30:70 (w/w) was applied, and sewn with silk thread. Experimental animals were terminated by decapitation. After the experimental animal died, jaw resection was performed to see the number of osteoblasts according to the predetermined observation day. The P1 group was observed on the 7th day, the P2 group was observed on the 14th day, and the P3 group was observed on the 21st day. The K1 group was observed on the 7th day, the K2 group was observed on the 14th day, and the K3 group was observed on the 21st day.

The resected bone tissue was then washed with a solution of Phosphate-buffered Saline (PBS) and followed by fixation in 10% buffered formalin solution for 1x24 hours. The fixed tissue was then decalcified and given a 10% Ethylene Diamine Tetra Acetic (EDTA) solution by changing the solution every day for 30 days.

After the bone tissue had softened, it was dehydrated in a solution of 70% alcohol for 1 hour, 80% alcohol for 1 hour, 90% alcohol for 1 hour, and 100% alcohol for 1 hour, and repeated 3 times. The clearing was carried out with Xylol 3 repetitions within 1 hour, 2 hours, and 3 hours respectively. The tissues were soaked in thawed paraffin at 60˚C for 2 hours and repeated 2 times. Embedding is carried out by heating paraffin at 60˚C which will be poured into the mold with bone tissue and then cooled until it hardens to form a paraffin block. Slicing was carried out on the paraffin block with a rotary microtome with a thickness of 4μ=4x10^{-3}, a water bath was carried out at 40˚C for 30 seconds, the tissue was dried and placed on an object glass then the paraffin on the preparation was melted with a hotplate at 60˚C for 30

minutes. Deparaffinized with xylol solution for 3 x 7 minutes, washed with 99% alcohol solution for 3 minutes and repeated, 90% alcohol for 3 minutes, 80% alcohol for 3 minutes, and 70% alcohol for 3 minutes, and washed with water running for 5 minutes.

Stained with Mayer Hematoxylin dye for 5 minutes, then rinsed with water for 7 minutes and continued with eosin staining for 10 seconds, then washed using alcohol 99%, 90%, 80%, 70% each for 2 minutes, washed, dried then given xylol for 2 minutes. After that, mounting is carried out with a cover glass that has been dripped with entellan or Canadian balsam.

Counting the number of osteoblasts was carried out using a microscope with a magnification of 400x for taking pictures and 1000x for 5 fields of view for counting, recording, and calculating the average of each cell. Data analysis was carried out using a one-way ANOVA test with a significance level of 5% and continued with the Tukey HSD posthoc test if there was a significant difference.

3. Results

Data were obtained from sample readings using a light microscope with a magnification of 1000x as many as 5 fields of view for calculation. The mean of the results of calculating the number of osteoblasts in the sample preparations for each group is shown in the Table 1.

Table 1 Average Number of Osteoblasts

Group	n	Average Number	Standard Deviation
K1	4	28.4	4.191
K2	4	19.15	1.652
K3	4	31.15	1.330
P1	4	28.40	0.490
P2	4	40.85	4.090
P3	4	18.95	2.932

Description: K1 : The control group received tooth extraction treatment and the number of osteoblasts was observed on the 7th day; K2 : The control group received tooth extraction treatment and the number of osteoblasts was observed on the 14th day; K3 : The control group received tooth extraction treatment and the number of osteoblasts was observed on the 21st day; P1 : The group that after extraction received the scaffold application treatment then the number of osteoblasts was observed on the 7th day; P2 : The group that after extraction received the scaffold application treatment then the number of osteoblasts was observed on the 14th day; P3 : The group that after extraction received the scaffold application treatment then the number of osteoblasts was observed on the 21st day

In the average table, group P2 showed the highest average number of osteoblasts compared to other groups. The lowest average number of osteoblasts was in the P3 group. Preparations were taken with 400x magnification. Pictures of the results of the preparation photos can be seen in Figure 1:

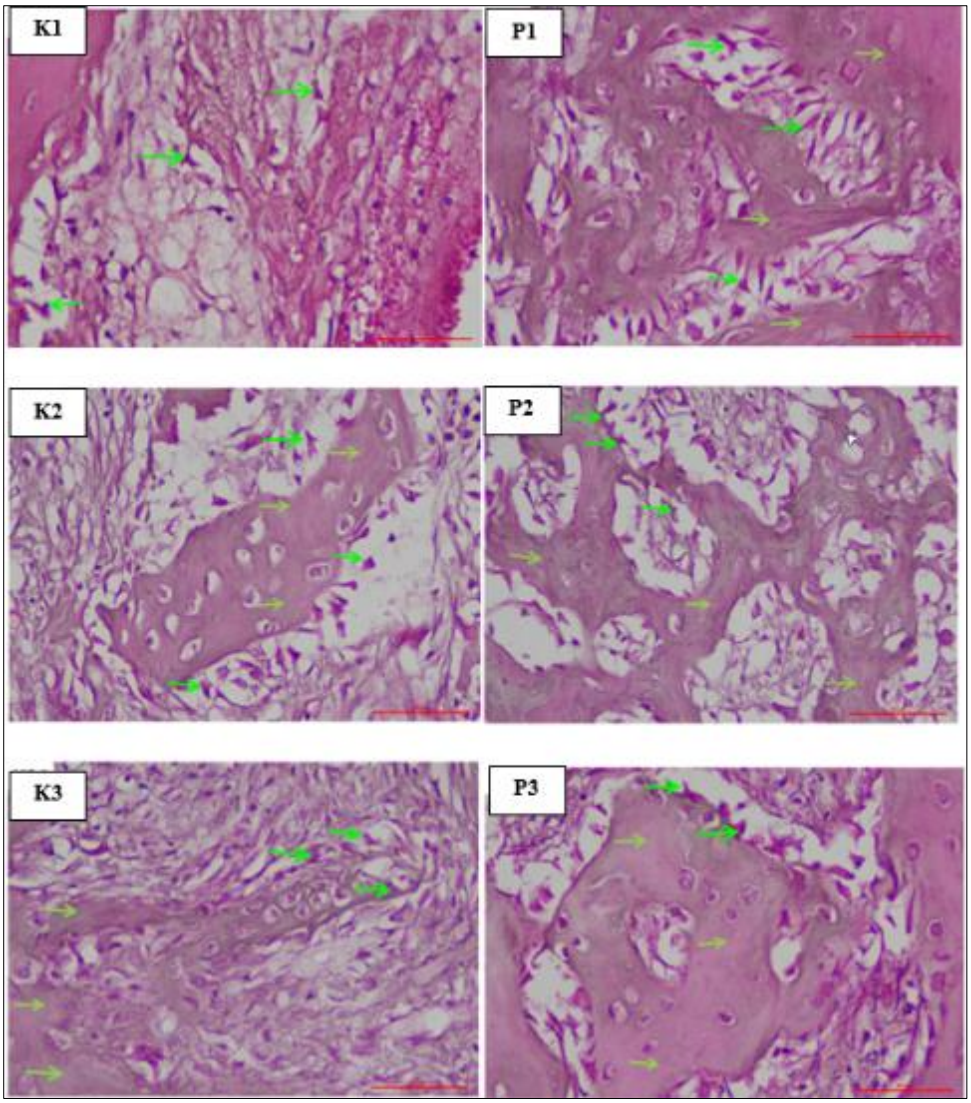

Figure 1 Examination results osteoblast cells in the control and treatment groups on days 7, 14 and 21

To find out the average data position for each sample group can be explained in the Figure 2.

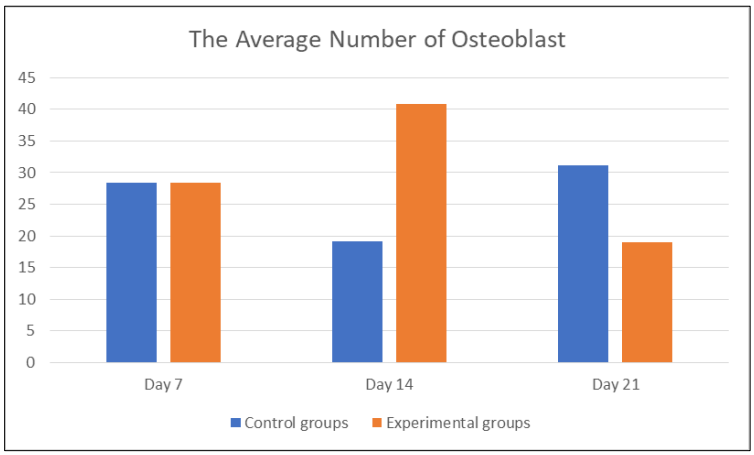

Figure 2 Diagram Showing The Average Number of Osteoblasts

The results of the one-way ANOVA comparative test showed a value of p = 0 (p <0.05), so it could be concluded that there was a significant difference between the control group and the treatment group. Tukey HSD posthoc test was carried out and the following results were obtained (Table 2).

Table 2 Tukey HSD Post-hoc Test Results Number of Osteoblasts

Number of Osteblasts	P3	P2	P1	K3	K2	K1
K1	0,006*	0*	1	0.795	0.006*	-
K2	1	0*	0.005*	0*	-	
K3	0*	0.003*	0.851	-		
P1	0.005*	0*	-			
P2	0*	-				
P3	-					

Annotation:
*= There is a significant difference (p value < α (0.05))

The P1 group and the K1 group showed p = 1 (p>0.05), which means that the difference in the number of osteoblasts between the P1 and K1 groups was not significant. In the P2 group and the K2 group, the value of P = 0 (P <0.05) was obtained, which meant that there was a significant difference. Group P3 and group K3, got a value of P = 0 (P <0.05), which means that there is a significant difference.

4. Discussion

In this study, a chitosan-hydroxyapatite carbonate gelatin scaffold was applied with a ratio of 30:70 (w/w) to the socket of Wistar rats (*Rattus norvegicus*) and the number of osteoblasts was observed on days 7, 14, and 21 to determine the effect of the scaffold on the number of osteoblasts. Based on the results obtained from this study, there was a significant difference in the number of osteoblasts in the treatment group compared to the control group. These findings support the hypothesis that the application of a chitosan-gelatin carbonate hydroxyapatite scaffold can help the process of bone regeneration by increasing the number of osteoblasts. Based on previous studies, chitosan plays a role in the bone healing process by increasing the infiltration of inflammatory cells in the trauma area and releasing growth factors. Chitosan can increase osteoblast migration and differentiation, and indirectly facilitate the process[21]. The N-acetylglucosamine component in chitosan which is also found in glycosaminoglycans supports the ability of chitosan to interact with growth factors, receptors, and adhesion proteins[22].

In this study, the results showed that the application of the C-G:CHA scaffold could increase the number of osteoblasts on days 7 and 14. This could also be caused by the presence of gelatin in the C-G:CHA scaffold. Gelatin is a promising choice of scaffold material, with good therapeutic and regenerative properties due to its similarity in chemical structure to the extracellular matrix[23]. Gelatin plays a role in increasing the number of osteoblasts by increasing cell adhesion and interactions[1]. Gelatin has a series of amino arginine-glycineaspartic acid (RGD), which is also present in collagen, and can increase cell adhesion, proliferation, migration, and differentiation[24,25].

Hydroxyapatite carbonate is often the material of choice for use in bone healing because it can be absorbed more quickly by osteoclasts to be replaced by new, better bone tissue. Hydroxyapatite carbonate also has osteoconductive and bioresorbable properties. Several studies explain that in observations made 7 days after the application of hydroxyapatite carbonate to the post-extraction rat tooth socket, there was osteoid deposition around the hydroxyapatite carbonate grains which proves that hydroxyapatite carbonate has osteoconductive properties[26,27].

The application of the chitosan-gelatin carbonate hydroxyapatite scaffold is thought to function as a medium to support the bone healing process, due to its ability to facilitate the growth of mesenchymal stromal cells (MSC), support tissue growth and development which acts as an extracellular matrix, cell adhesion for proliferation and differentiation to form new tissues[2].

The largest average increase in the number of osteoblasts can be seen in the P2 group. This might have happened due to the application of the chitosan-gelatin carbonate hydroxyapatite scaffold which helped increase cell adhesion and proliferation during the bone regeneration process[28]. On day 14, which was the day of observing the number of

osteoblasts in group P2, there was a peak in the process of hard callus formation[20]. During the process of hard callus formation, RANK-L would be expressed thereby stimulating further differentiation of chondroblasts, chondroclasts, osteoblasts, and osteoclasts[29]. The increase in the number of osteoblasts on day 14 can also be influenced by hydroxyapatite carbonate. Biomaterials containing calcium phosphates such as hydroxyapatite, tricalcium phosphate (TCP), and biphasic calcium phosphate (BCP) are good candidates to assist bone reconstruction due to their osteoconductive properties[20]. High concentrations of calcium and phosphate ions can increase the bone mineral formation and affect the expression of osteogenic genes that play a role in osteoblast differentiation such as RUNX2 and BMP[31].

The smallest average number of osteoblasts was in the P3 treatment group, which was observed on the 21st day after scaffold application. The decrease in the number of osteoblasts on day 21 after the application of the CG:CHA scaffold was in line with previous studies, which stated that the number of osteoblasts indicated an ongoing process of bone formation. After the maturation process, osteoblasts will calcify to form osteocytes which will then go through a remodeling process[15]. On the 21st day, the remodeling process takes place, namely the bone regeneration phase when a balance begins to occur between the activities of osteoclasts whose job is to absorb, and osteoblasts which are depositing new bone tissue[7,20]. This could be the reason why the number of osteoblasts on day 21 after the application of the C-G:CHA scaffold decreased. The application of the C-G:CHA scaffold is expected to accelerate the remodeling phase due to the combination of the three ingredients, namely chitosan-gelatin carbonate hydroxyapatite which supports the bone healing process.

There was no significant difference in the mean number of osteoblasts between the K1 control group and the P1 treatment group. This might happen because the osteoblast differentiation stage is still ongoing, even though the 7th day is predicted to be the peak of young callus formation[20]. In the early stages of osteoblast differentiation, callus fibrocartilage begins to form due to angiogenesis triggered by communication between cells and VEGF on days 5 to 11 which marks the start of the bone repair phase. Granulation tissue rich in fibrin begins to develop, and MSCs that are recruited to the bone defect area will begin to differentiate due to the influence of BMP to form fibroblasts, chondroblasts, and osteoblasts[29]. In the group that received the treatment in the form of C-G:CHA scaffold application, there was an increase in the number of osteoblasts on days 7 and 14, with a decrease in the number of osteoblasts on day 21. Similar results were also found in several previous studies. In a study conducted by Dewi & Triawan, histological evaluation of rat tibial defects showed that the application of apatite carbonate and apatite-chitosan carbonate resulted in a higher number of osteoblasts and osteoclasts than the control group 7 days after implantation of the apatite-chitosan carbonate scaffold, but the opposite was 14th and 21st. The number of osteoblasts was lower than in the control group on days 14th and 21st after scaffold implantation, indicating that osteoblasts calcified faster[15]. In a previous study, chitosan was used in a gel dosage form to determine the expression of BMP- 2 on bone healing in Wistar rats (*Rattus norvegicus*) after tooth extraction. The results obtained were an increase in BMP-2 expression on days 7 and 14 and a decrease on days 21. As is known, BMP-2 is an important factor in the process of maturation and activity of osteoblasts, because of its ability to induce bone formation[21]. The findings in this study, which are supported by the studies mentioned above indicate that the application of scaffolds with chitosan-gelatin carbonate as the basis hydroxyapatite (C-G:CHA) can accelerate the process of bone regeneration.

5. Conclusion

The application of chitosan-gelatin carbonate hydroxyapatite (C-G:CHA) scaffold with a ratio of 30:70 (w/w) increased the number of osteoblasts on days 7, and 14 after tooth extraction. The application of a scaffold with the basic ingredients of chitosan-gelatin carbonate hydroxyapatite (C-G:CHA) can accelerate the process of bone regeneration.

Compliance with ethical standards

Acknowledgments

The authors are thankful to Research and Innovation Institute and Rector of Universitas Airlangga, Indonesia for the foreign partner collaborative research grant No. 212/UN3/2021.

Disclosure of conflict of interest

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

Statement of ethical approval

This research met ethical requirements and was approved by the UNAIR Faculty of Dentistry Ethics Committee No. 560/HRECC.FODM/VIII/2022.

References

[1] Moro, JS, Barcelos, RCS, Terra, TG, Danesi, CC, 'Tissue Engineering Perspectives in dentistry: review of the literature', Revista Gaucha de Odontologia, 2018; vol. 66, no. 4. pp. 361-367.

[2] Bachtiar, EW, Amir, LR, Suhardi, P, & Abas, B, 'Scaffold degradation during bone tissue reconstruction in Macaca nemestrina mandible', Interventional Medicine and Applied Science, 2016; vol. 8, no. 2, pp. 77-81.

[3] Kubilius, M, Kubilius, R, & Gleiznys, A, 'The Preservation of Alveolar Bone Ridge during Tooth Extraction', Stomatologija, Baltic Dental and Maxillofacial Journal, 2012; vol. 14, no. 1, pp. 3-11.

[4] Liu, J, & Kerns, DG, 'Mechanisms of Guided Bone Regeneration: A Review', The Open Dentistry Journal, 2014; vol. 8, pp. 56-65.

[5] Pagni, G, Pellegrini, G, Giannobile, WV, & Rasperini, G, 'Postextraction Alveolar Ridge Preservation: Biological Basis and Treatments', International Journal of Dentistry, 2012; vol. 2012. pp. 1-13.

[6] Tomlin, EM, Nelson, SJ, & Rossmann, JA, 'Ridge Preservation for Implant Therapy: A Review of the Literature', The Open Dentistry Journal, 2014; vol. 8, pp. 66-76.

[7] Maruyama, M, Rhee, C, Utsunomiya, T, Zhang, N, Ueno, M, Yao, Z, & Goodman, SB, 'Modulation of the Inflammatory Response and Bone Healing', Frontiers in Endocrinology, 2020; vol. 11, no. 386, pp. 1-14.

[8] Lin, X, Patil, S, Gao, YG, & Qian, A, 'The Bone Extracellular Matrix in Bone Formation and Regeneration', Frontiers in Pharmacology, 2020; vol. 11, no. 757, pp. 1-15.

[9] Ansari, M, 'Bone tissue regeneration: biology, strategies and interface studies', Progress in Biomaterials, 2019; vol. 8, pp. 223-237.

[10] Jimi, E, Hirata, S, Osawa, K, Terashita, M, Kitamura, C, & Fukushima, H, 'The Current and Future Theraphies of Bone Regeneration to Repair Bone Defects', International Journal of Dentistry, 2012; vol. 2012, pp. 1-7.

[11] Murphy, CM, O'Brien, FJO, Little, DG & Schindeler, A, 'Cell-Scaffold Interactions in The Bone Tissue Engineering', European Cells and Materials, 2013; vol. 26, pp. 120-132.

[12] O'Brien, FJ, 'Biomaterials & Scaffolds for Tissue Engineering', Materials Today, 2011; vol. 14, no. 3 pp. 88-95.

[13] Tripathi, G, & Basu, B, 'A Porous Hydroxyapatite Scaffold for Bone Tissue Engineering: Physico-mechanical and Biological Evaluations', Ceramics International, 2012; vol. 38, pp. 341-349.

[14] Yazid, F, Nurmimie, A, Nur, AL, Shahrul, HZA, & Rohaya, M, 'Scaffold Selection for Tissue Engineering in Dentistry', Med & Health, 2020; vol. 15, no. 1, pp. 34-53.

[15] Dewi, AH, & Triawan, A, 'The Newly Bone Formation with Carbonate Apatie Chitosan Bone Substitute in the Rat Tibia', The Indonesian J Dent Res, 2011; vol. 11, no. 3, pp. 154-160.

[16] Yuliati, A, Merlindika, Y, Munadziroh, E, Ari, M, Fadhlallah, M, Rianti, D, Maretaningtias, DA, & Kartikasari, N, 'Mechanical Strength and Porosity of Carbonate Apatite-Chitosan-Gelatine Scaffold in Various Ratio as a Biomaterial Candidate in Tissue Engineering', Key Engineering Materials, 2019; vol. 829, pp. 173-181

[17] Li, Y, Chen, S, Li, L, Qin, L, Wang, X, & Lai, Y, 'Bone Defect Animal Models for Testing Efficacy of Bone Substitute Biomaterials', Journal of Orthopedic Translation, 2015; vol. 3, no. 3, pp. 95-104.

[18] Ariani, MD, 'Novel development of carbonate apatite-chitosan scaffolds based on lyophilization technique for bone tissue engineering', Dental Journal, 2012; vol. 45, no. 3, pp. 150-155.

[19] Sularsih, 'The Pore Size of Chitosan-Aloe Vera Scaffold and its Effect on VEGF Expressions and Woven Alveolar Bone Healing of Tooth Extraction of Cavia Cobaya', Dental Journal, 2020; vol. 53, no. 3, pp. 115-121.

[20] Marsell, R, & Einhorn, TA, 'The biology of fracture healing', Injury International Journal of the Care of the Injured, 2011; vol. 42, no. 6, pp. 551-555.

[21] Sularsih, & Wahjuningsih, E, 'Expression of bone morphogenetic protein-2 after using chitosan gel with different molecular weight on wound healing process of dental extraction', Dental Journal, 2015; vol. 48, no. 2, pp. 53-58.

[22] Maji, K, Dasgupta, S, Kundu, B, Bissoyi, A, 'Development of Gelatin-Chitosan-Hydroxyapatite based bioactive bone scaffold with controlled pore size and mechanical strength', Journal of Biomaterials Science Polymer Edition, 2015; vol. 26, pp. 1-20.

[23] Afewerki, S, Sheikhi, A, Kannan, S, Ahadian, S, & Khademhosseini, A, 'Gelatin-polysaccharide composite scaffolds for 3D cell culture and tissue engineering: Towards natural therapeutics', Bioengineering & Translational Medicine, 2019; vol. 4, no. 1, pp. 96-116.

[24] Georgopoulou, A, Papadogiannis, F, Batsali, A, Marakis, J, Alpantaki, K, Eliopoulos, A, Pontikoglou, C & Chatzinikolaidou, M, 'Chitosan/gelatin scaffolds support bone regeneration', Journal of Materials Science: Materials in Medicine, 2017; vol. 29, no. 5, pp. 1-13.

[25] Kartikasari, N, Yuliati, A, Listiana, I, Setijanto, D, Suardita, S, Ariani, DA, Sosiawan, A, 'Characteristic of Bovine Hydroxyapatite-Gelatin-Chitosan Scaffolds as Biomaterial Candidate for Bone Tissue Engneering', IEEE EMBS Conference on Biomedical Engineering and Sciences, 2016; pp. 623-626.

[26] Calassans-Maia, MD, Melo, BR, Alves, ATNN, Resende, RFB, Louro, RS, Sartoretto SC, Granjeiro, JM, & Alves, GG, 'Cytocompatibility and Biocompatibility of Nanostructured Carbonated Hydroxyapatite Spheres for Bone Repair', J Appl Oral Sci, 2015; vol. 23, no. 6, pp. 599-608.

[27] Setiawatie, EM, Widyanti, P, Ryan, M, & Rubianto, M, 'Carbonate Hydroxyapatite-Hyaluronic Acid as Bone Healing Accelerator: In vitro and In-vivo Studies on the Alveolar Bone of Wistar Rats', Journal of International Dental and Medical Research, 2019; vol. 12, no. 4, pp. 1280-1286.

[28] Zhu G, Zhang T, Chen M, Yao K, Huang X, Zhang B, Li Y, Liu J, Wang Y, & Zhao Z, 'Bone Physiological Microenvironment and Healing Mechanism: Basis for Future Bone-Tissue Engineering Scaffolds', Bioactive Materials, 2021; vol. 6, no. 2021, pp. 4110-4140.

[29] Sheen, JR & Garla, VV, 'Fracture Healing Overview', StatPearls, 2022; pp. 2-5. viewed 19 March 2022 https://www.ncbi.nlm.nih.gov/books/NBK551678/

[30] Lu, J, Yu, H, & Chen, C, 'Biological Properties of Calcium Phosphate Biomaterials for Bone Repair: a Review', Royal Society of Chemistry Advances, 2018; vol. 2018, no. 8, pp. 2015-2033.

[31] Hou, X, Zhan, L, Zhou, Z, Luo, X, Wang, T, Zhao, X, Lu, B, Chen, F, & Zheng, L, 'Calcium Phosphate-Based Biomaterials for Bone Repair', Journal of Functional Biomaterials, 2022; vol. 13, no. 187, pp. 1-39.