Enhanced Osteoblasts and Collagen Production Using a Blood Cockle (Anadara granosa) and Lemuru Fish Oil (Sardinella longiceps) Granule Combination for Tooth Socket Healing

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Abstract

This study investigated the efficacy of a granule combination comprising blood cockle shell powder (Anadara granosa) and Lemuru fish oil (Sardinella longiceps) in accelerating postextraction tooth-socket healing, with osteoblasts and collagen as the key parameters.

Forty Wistar rats were divided into five groups: a control group receiving no treatment, a group administered A. granosa powder (treatment 1), and the remaining groups receiving combinations of A. granosa and Lemuru fish oil granules at concentrations of 10%, 20%, and 30% (treatments 2, 3, and 4), respectively. All rats underwent intentional extraction of their lower right first molar in the mandible. After 14 days, the rats were sacrificed, and their mandibles were transversally sectioned. Masson trichrome staining was used for collagen analysis, whereas hematoxylin and eosin staining was used for osteoblast evaluation. Light microscopy was used for observations. Data were analyzed using one-way ANOVA for osteoblasts and descriptive statistics and Mann–Whitney tests for collagen, with statistical significance set at $p < 0.05$. The average osteoblast count significantly increased in the experimental groups compared with the control group ($p < 0.05$). These counts were 6.901 in the control, 8.760 in treatment 1, 9.758 in treatment 2, 11.568 in treatment 3, and 19.950 in treatment 4. Collagen density scores for each group were as follows: control = 1; treatment 1 = 1; treatment 2 = 2; treatment 3 = 3; and treatment 4 = 2 Mann–Whitney tests revealed significant differences in collagen density between the groups treated with the A. granosa and Lemuru fish oil combination at 20% ($p = 0.025$) and 30% ($p = 0.041$) concentrations.

The combination of A. granosa and Lemuru fish oil in granules significantly enhances tooth-socket healing when used as a postextraction treatment in rats.

Experimental article (J Int Dent Med Res 2024; 17(1): 58-63)

Keywords: Anadara Granosa, Sardinella Longiceps Oil, Collagen, Osteoblasts, Socket Healing.

Received date: 30 September 2023 **Accept date:** 05 January 2024

Introduction

The tooth extraction rate in Indonesia remains notably high, leading to postextraction complications. According to the 2013 National Basic Health Research (RISKESDAS), the national Decayed Missing and Filled Teeth index indicates a tooth decay severity of with a missing teeth component value of [1,2] This signifies that 290 tooth roots per 100 individuals either undergo permanent extraction or remain unaddressed. Alveolar bone resorption, which occurs in nearly 90% of tooth extractions[1], poses challenges for dental implantation and prosthodontic treatments[3]. Moreover, prolonged socket healing affects post-tooth extraction care[4]. The present study aims to address alveolar bone resorption and socket healing challenges, and explore effective bone-like materials.

Socket healing is a natural process following tooth extraction, encompassing bone healing. Shortly after tooth extraction, vascular contractions lead to blood clot formation in the

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socket, comprising erythrocytes and leukocytes in fibrin threads. Approximately one week after tooth extraction, the blood clot–filled socket is predominantly replaced by granulation tissue rich in vascular structures, inflammatory cells, and erythrocytes. Days 3–14 mark angiogenesis, characterized by fibroblast migration and capillary formation[5]. Fibroblasts and angiogenesis play pivotal roles in vascular growth, facilitated by various factors, including FGF, VEGF, PDGF, Angiodenin, TGF-α, and TGF-β [6]. Of these, TGF-β increases the number of fibroblasts and preosteoblasts, promoting their maturation into osteoblasts [7].

Osteoblast cells emerge around the defect area within the bone cortex [8]. They secrete organic collagen and noncollagen (bone matrix) components, regulating mineralization involving calcium phosphate, which forms osteoid and contributes to bone formation. Collagen is detected as early as day three after injury and increases until the third week. Mature osteoblasts produce bone matrix, primarily type I collagen, regulating newly formed bone mineralization [9]. Within 6–8 weeks, provisional matrix and woven bone replace all granulation tissue [10].

Effective bone-like materials are employed to restore damaged bone tissue, necessitating the structural and property congruence of these materials with bone to ensure accelerated healing. Various types and tissue sources promote socket healing [11]. Bone materials must exhibit biological mechanisms, such as osteoconduction, osteoinduction, osteointegration, and osteogenesis [11]. Ceramic grafts, including calcium carbonate ($CaCO_3$), hydroxyapatite, calcium sulfate, and tricalcium phosphate, are commonly used [11].

Using readily available waste shells, such as those from blood cockles (*Anadara granosa*), is common in promoting socket healing [12]. Blood cockle shells naturally contain $CaCO_3$ in the polymorphic aragonite phase, constituting 95%–98% of their composition [13], indicating their potential as a bone-like material, given the similarity of their composition to that of bone [13]. Mineral content in blood clam shells includes 98.70% $CaCO_3$, 0.05% Mg, 0.90% Na, 0.02% P, and 0.20% other minerals[14]. $CaCO_3$ exhibits strong osteoconductive properties, excellent adhesion to periodontal ligaments, potential structural mimicry, enhanced osteoblast differentiation, and nontransmission of diseases[13,14].

Lemuru (*Sardinella longiceps*), also known as Indian oil sardine, is a fish rich in omega-3 fatty acids.[15] Omega-3 in fish oil plays a crucial role in regulating inflammation during bone formation.[16] Lemuru fish oil contains n-3 polyunsaturated fatty acids (PUFA) about 33.7%, with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels at 14.5% and 13.2%, respectively.[16] These n-3 PUFAs serve as eicosanoid precursors involved in bone metabolism as well as prostaglandin and leukotriene production. EPA and DHA in diets replace n-6 PUFAs, reducing IL-1, IL-6, and TNF-α content, thereby decreasing proinflammatory cytokine levels, promoting bone formation, and mitigating bone destruction.[16] Indeed, EPA and DHA can effectively reduce production inflammatory mediators and bone resorption. Fish oil increases bone sialoprotein (BSP) expression, impacting osteoblast activity while decreasing osteopontin levels and affecting osteoclast activity. Successful bone remodeling necessitates reduced bone resorption and mineral balance in the bone, regulated by osteoblasts. Osteoblasts secrete BSP, facilitating osteoblast motility toward mineralized bone regions. Mature osteoblasts produce osteoid, type I collagen, growth factors, and alkaline phosphate.[17,18]

This study aims to explore the potential of using natural materials, specifically $CaCO_3$-rich blood cockle shells and omega-3–rich Lemuru fish oil, to enhance socket healing and bone formation, thereby promoting socket healing following tooth extraction.

Materials and methods

This experimental research, conducted in a laboratory, involved using a sample of Wistar rats (n = 40) aged 3-3,5 months and weighed 250-300 grams.[18] Sample divided into five groups: a control group receiving no treatment, a group treated with blood cockle shell powder (treatment 1), and three groups treated with a combination of blood cockle shell and Lemuru fish oil gel granules at concentrations of 10%, 20%, and 30% (treatments 2, 3, and 4, respectively). The rats, after acclimatization and grouping, underwent tooth extractions in the mandibular right side. Subsequently, any

remaining tissue in the socket was meticulously removed using a small excavator, followed by constant irrigation with a sterile salt solution before the application of granules. The sockets were then sutured. After 14 days, the rats were euthanized, and their right mandibles were transversely sectioned using scissors and a scalpel. These mandibular sections were immersed in a 10% buffered formalin solution, with osteoblasts examined using hematoxylin and eosin staining and collagen analysis conducted using Masson trichrome (MT) staining during the preparation of histopathological slides. Observations were performed using a light microscope.

Results

The study data were subjected to descriptive analysis to provide an overview of data distribution and for data summarization, aiding in the presentation of research findings.

Group	Mean ± standard deviation
C	6.901 ± 1.7734
EP 1	8.760 ± 1.1812
EP 2	9.758 ± 0.4983
EP 3	11.568 ± 0.8968
EP 4	19.950 ± 1.1286

EP1: *Anadara granosa* alone.
EP 2: combination of *A. granosa* and Lemuru fish oil with a 10% concentration.
EP 3: combination of *A. granosa* and Lemuru fish oil with a 20% concentration.
EP 4: combination of *A. granosa* and Lemuru fish oil with a 30% concentration.

Table 1. Mean number of osteoblasts in the socket healing process on day 14.

Group	P1	P2	P3	P4
C	.006*	.000*	.000*	.000*
EP 1		.121	.000*	.000*
EP 2			.007*	.000*
EP 3				.000*

*indicates significant differences.
EP1: *Anadara granosa* alone.
EP 2: combination of *A. granosa* and Lemuru fish oil with a 10% concentration.
EP 3: combination of *A. granosa* and Lemuru fish oil with a 20% concentration.
EP 4: combination of *A. granosa* and Lemuru fish oil with a 30% concentration.

Table 2. Results of post-hoc least significant difference test.

Table 2 shows the outcomes of the post-hoc least significant difference test, revealing significant differences in osteoblast counts ($p < 0.05$) across all groups except for the comparison between the groups with *A. granosa* alone and the combination of *A. granosa* and Lemuru fish oil with a 10% concentration. These results highlight the efficacy of the administered granules in enhancing osteoblast cell numbers on day 14 of bone healing.

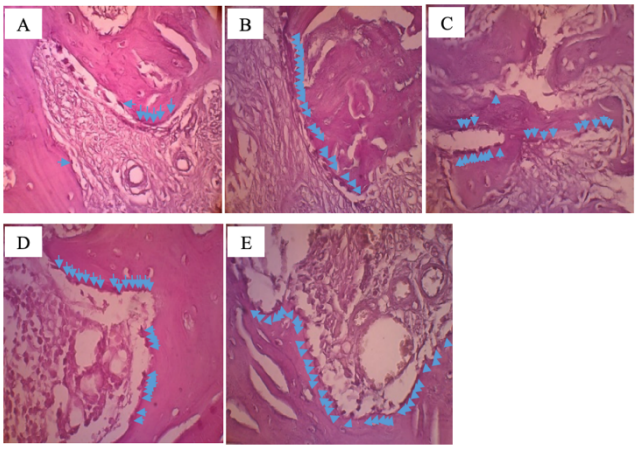

Figure 1. Microscopic depiction of osteoblasts cell through staining with Hematoxylin eosin: A Control group B EP1 group C EP2 group D EP3 group E EP4 group. Blue arrow show osteoblasts cell.

Group	Modus/Mean
C	1
EP 1	1
EP 2	2
EP 3	3
EP 4	1

Table 3. Modus/median collagen density score on day 14.

Group	P1	P2	P3	P4
C	.532	.104	.025*	.041*
EP 1		.343	.115	.193
EP 2			.475	.775
EP 3				.606

Table 4. Results of the Mann–Whitney test.
*indicates significant differences.

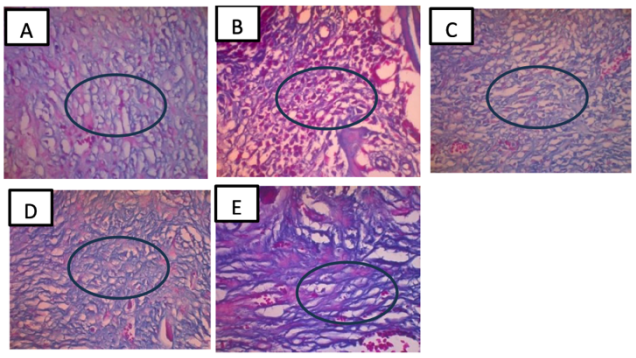

Figure 2. Microscopic depiction of collagen density through staining with Masson trichrome. A: Control group (score 11); B: EP1 group (score 11); C: EP2 group (score 22); D: EP3 group (score 3); E: EP4 group (score 2).

Discussion

This study aimed to elucidate the role of granules comprised of blood cockle shells (*A. granosa*) and Lemuru fish oil (*S. longiceps*) in tooth-socket healing in Wistar rats. Lemuru fish oil, rich in n-3 PUFAs, particularly EPA and DHA, reduces osteoblast cell apoptosis. Dietary EPA and DHA, by replacing n-6 PUFAs, diminish IL-1, IL-6, and TNF-α levels, leading to decreased proinflammatory cytokine content, increased bone formation, and reduced bone destruction. Decreases in proinflammatory cytokine levels are associated with a reduction in osteoclasts and an increase in osteoblasts.[16,17]

In this study, hematoxylin and eosin and MT staining were used for histological observations and assessing collagen synthesis levels. This methodology aligns with the approach taken by Majid et al. in their research on wound dressings. Similarly, Omar et al. used MT staining to assess collagen synthesis levels, compare re-epithelialization rates across different groups, and evaluate epithelial gaps.[19,20]

The alveolar bone healing process following tooth extraction typically begins with hemostasis and the formation of blood clots.[6] During the inflammatory phase, macrophages are activated, leading to the release of proinflammatory cytokines, such as TNF-α, IL-1, IL-1β, and IL-6, alongside antiinflammatory cytokines, including IL-4, IL-5, IL-10, and IL-13.[6] The peak of the inflammatory response is reached within 48 hours, with full completion occurring approximately one week after extraction. The proliferation phase ensues as cytokines and growth factors released during the inflammatory phase stimulate fibroblast proliferation, extracellular matrix formation, and calcium salt attachment. Abundant collagen, crucial for connecting the formed bone gaps, is required in the fibroplasia phase.[8] In the remodeling phase, collagen type III is replaced by more robust type I collagen, improving tissue strain resilience.[8]

Collagen plays a pivotal role at each stage of healing, contributing to hemostasis, increased cellular component levels, and heightened growth factor activity.[21] Collagen is an important factor for socket healing process because type I collagen forms more than 90% of the organic mass of bone. It provides integrity and strength to connective tissue.[22] Mature osteoblasts are central in synthesizing bone matrix, particularly type I collagen, and regulating the mineralization of newly formed bone.[8,9] Type I collagen undergoes mineralization to form the bone matrix, gradually enclosing bone fragment ends within a fusiform mass containing woven bone.[21]

Blood cockle shells serve as viable bone grafts owing to their $CaCO_3$ content, giving them a similar composition to bone.[13] $CaCO_3$ crystals derived from these shells facilitate osteoblast proliferation, differentiation, and adhesion, enhancing osteoblast function.[23] This process exhibits increased osteoblast differentiation, leading to the secretion of type I collagen.[17] Crystalline $CaCO_3$, in combination with type I collagen, shows strong osteoconductive properties and promotes BMP-2 activity, consequently stimulating bone regeneration, repair, and osteoblast function.[8,24] BMP-2 helps osteoblast differentiation and proliferation, to encourage mesenchymal cells to proliferate and differentiate into active osteoblast cells so that they can improve the quality of alveolar bone.[25] When blood cockle shells are combined with Lemuru fish oil, proinflammatory cytokine production decreases, suppressing osteoblast apoptosis and promoting osteoblast proliferation, which, in turn, enhances collagen secretion.[16,26]

Group K is a group with normal bone healing, where the number of osteoblasts in this group is still visible even without treatment. The socket healing process can be divided into four phases and each phase is differentiated according to the presence of different cells and extracellular matrix components. This phase starts from the hemostasis phase, inflammatory phase, reparative phase and remodeling phase.[26] The inflammatory phase is the most important phase in socket healing which consists of acute inflammation turning into chronic inflammation and will form granulation tissue that closes the socket.[27]

Group P1 was the group that was given bone graft from blood cockle shells (Anadara granosa) containing calcium carbonate. Blood cockle shells (Anadara granosa) have been proven to be useful in bone healing treatment, cause the structure of shellfish is generally similar to bone and the mechanical properties resemble bone. Clam shells contain high levels of calcium carbonate and are biocompatible and

osteoconductive. Clam shells act as adequate carriers for growth factors and enable cell attachment, growth, spreading and cell differentiation. The P1 group did not have a significant difference in the number of osteoblasts compared to the P2 group, this happened because the P1 group was only given blood clam shell bone graft so it was less efficient in the bone formation process. The addition of lemuru fish oil containing EPA and DHA to the graft material can inhibit osteoblast cell apoptosis, so that the number of osteoblast cells increases and osteoclast cells decrease.[16,27] In addition, experimental animals in group P2 experienced molar root fractures during treatment which resulted in the socket healing process not happened well. Healthy granulation tissue and osteoblast cells cannot form completely because they are still experiencing a longer inflammatory phase.[28]

Groups P2, P3, P4 are groups that were given a combination of blood cockle shell (Anadara granosa) bone graft containing calcium carbonate with Lemuru fish oil gel (Sardinella longiceps) in the form of a scaffold containing omega-3 with concentrations of 10%, 20% and 30%. %. Calcium carbonate nanocrystals derived from shellfish can facilitate the proliferation and apoptosis of osteoblast cells. This shows that osteoblast differentiation also increases, such as which is indicated by increased osteogenic alkaline phosphatase activity, protein synthesis and extracellular calcium deposition. The osteogenic activity of alkaline phosphatase was enhanced by CaCO3 nanocrystals. Increased osteogenic activity of alkaline phosphatase increases the differentiation of mesenchymal cells.[29]

In groups P2, P3 and P4, the formation of new bone in the socket occurred well but was not optimal because the experimental animals were only observed until day 14, where on day 14 the reparative phase which created new bone did not yet appear optimal. New bone is formed due to the administration of a combination of blood clam shell bone graft and lemuru fish oil gel which has been applied in the form of a scaffold, so that it can become a medium for stem cells and osteoblasts to attach, live and develop well in the socket. Scaffolds also help the formation of blood vessels in the formation of new bone better.[30] Stimulation from bone graft is expected to increase cell biological activity and the body can adapt well. During bone healing, bone graft will stimulate and cause osteoblasts to proliferate more to migrate to the socket area.[31]

Conclusions

The combination of blood cockle shell (*A. granosa*) granules with Lemuru fish oil (*S. longiceps*) gel markedly accelerates socket healing, as evidenced by increased osteoblast cell counts and collagen density.

Acknowledgements

The Author would like to thank especially Faculty of Dentistry Hang Tuah University where has supported this research.

Declaration of Interest

The authors report no conflict of interest.

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