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Investigation of 3% Binahong (*Anredera cordifolia*) Leaf Extract Nanogel on the Alveolar Bone Healing: BMP-2 Modulation in Rat Models

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A B S T R A C T

Background: Alveolar bone healing relies on osteoblasts activity and differentiation, with bone morphogenetic protein-2 (BMP-2) playing a crucial regulatory role. Binahong leaf extract (BLE) has demonstrated efficacy in bone healing due to its rich phytochemical composition. Nanogel offers enhanced bioavailability and targeted release to effectively deliver therapeutic agents. This study aims to assess the impact of 3% BLE nanogel on BMP-2 expression in tooth extraction socket healing. **Methods:** Thirty male Wistar rat underwent anaesthesia for the extraction of their lower incisors to induce alveolar bone healing. Subjects were randomly assigned into treatment (BLE nanogel) and control (base nanogel) group. Five rats from each group were respectively sacrificed at 7, 14, and 28 days after procedures. BMP-2 expression was assessed by performing immunohistochemistry analysis using BMP antibody reagent. Data were analysed using chi-square. **Results:** The analysis of BMP-2 expression showed no significant difference between treatment and control groups on days 7, 14, and 28 ($p>0,05$). However, a subtle increase was observed in the treatment group throughout observation period. **Conclusion:** Application of 3% BLE nanogel may enhance the expression of BMP-2, even though it was not significantly increase. The findings underscore the complex interplay between Binahong leaf extract, nanogel and BMP-2 expression.

1. Introduction

Preserving dental health requires the wellness of structures that provide support to the teeth, where the alveolar bone holds significant importance among these vital structures.[1] However, tooth extraction can disrupt alveolar bone architecture, leading to bone loss and compromising the stability of adjacent teeth.[2–4] Maintaining the stability and functionality of surrounding tissues relies heavily on the timely and effective regeneration of alveolar bone. Therefore, this process is essential for sustaining oral health, function, and aesthetics, making alveolar bone healing a critical aspect of dentistry.[5]

Alveolar bone healing is a complex and systematic process involving various cell types and chemical mediators, following the same four overlapped stages as general wound healing: hemostasis, inflammation, proliferation, and remodelling.[4,6,7] In the complex process of alveolar bone healing, bone morphogenetic protein-2 (BMP-2) plays a pivotal role as a key regulator of osteogenesis.[3,8] BMP-2 stimulates the differentiation of mesenchymal stem cells (MSC) into osteoblasts, the bone-forming cells essential for bone regeneration.[3,4,8] Osteoblasts are vital cells involved in alveolar bone healing. They produce the bone matrix and play a crucial role from the early stages of healing (proliferation) to later phases (remodelling).[6,7]

There is a growing need for alternative therapeutic approaches that can enhance bone healing and mitigate the risk of complications and morbidities.[6] *Anredera cordifolia*, known as Binahong, has been used traditionally as a wound-healing agent in Indonesia.[6,7,9] Its rich phytochemical composition, including flavonoids, phenolic compounds, saponins, and alkaloids exhibits anti-inflammatory, antioxidant, and osteogenic properties.[9–11] Notably, these studies have conducted in-depth molecular assessments, uncovering Binahong's capacity to modulate the expression of BMP-2 as one of the regulators in osteogenesis.[6,12]

Previous studies have investigated the effects of Binahong leaf extract and gel formulations on post-tooth extraction wound healing, and showed increased fibroblasts, osteoblasts, and osteocytes.[7,12–14] However, there remains a gap in understanding how modifying the formulation, particularly in the form of nanogels, may enhance therapeutic efficacy. Nanogels were introduced as a novel drug delivery system that offers a promising avenue for improving the efficacy of bioactive compounds like Binahong leaf extract.[15,16] There are advantages studied such as enhanced bioavailability, biodegradability, and targeted and controlled release, thereby enhancing therapeutic efficacy while minimizing adverse effects.[17,18]

This *in-vivo* study investigates the effect of 3% Binahong leaf extract nanogel on alveolar bone healing in post-tooth extraction, focusing on the modulation of BMP-2 expression. By elucidating the regulatory effects of Binahong leaf extract nanogel on BMP-2 expression, this research aims to provide insights into its potential as a therapeutic intervention for enhancing alveolar bone regeneration.

2. Methods

Ethical considerations

Experimental procedures conducted in this study adhered to the guidelines in the Institutional Animal Care and Usage Committee (IACUC), following the ARRIVE guidelines 2.0. Ethics approval was obtained from the Animal Research Ethics Committee (AREC), Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (USU) under the reference number: 0440/KEPH-FMIPA/2023.

Preparation of 3% Binahong leaf extract nanogel

The Binahong leaf utilized in this research was acquired from the Pharmacognosy Laboratory, Faculty of Pharmacy, USU, and has been identified in Herbarium Medanense (MEDA) USU.[19] Four hundred grams of fully mature Binahong leaves, approximately 12 weeks old, were picked and sourced from Simpang Perdagangan Village, Tigabinanga District, Karo Regency, North Sumatra, Indonesia.[7,9] In this study, Binahong leaves were extracted using the maceration method with 80% ethanol. Initially, the Binahong leaves were pulverized into powder using an electric blender (Philips HR2115, Indonesia) and then soaked in 80% ethanol in a sealed container at room temperature for five days. Then, the solvent was replaced with a fresh one and soaked for another 48 hours. The extract underwent a final filtration process and was then followed by solvent evaporation using a water bath until the extract was dried.[7,9,20] Binahong leaf extract has been studied for its toxicity effect on safety measures.[21]

The nanogel formulation obtained from the Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara from the modification of gel formulation used in previous studies.[20] The nanogel formulation of 3% Binahong leaf extract contains 3g ethanol extract of Binahong leaves, 0.625g Carbopol 940, 0.625g HPMC K4M, 10g glycerines, 1g TEA, 0.1g nipagin, 0.1g nipasol, and aquadest ad 100. This novel formulation has been evaluated for its stability.[19] Formulation of 3% Binahong leaf extract nanogel was ready to be applied to the extraction socket.

Animal model

The sample size for this study was determined by referencing prior research of a comparable nature.[7,12] In the previous study, the effect size (Cohen's d) between the experimental and control groups was

found to be 3.31.[12] Calculations yielded a minimum sample size of four animals, which was subsequently adjusted to five animals per group. There are 6 groups involved in this study, resulting in a total of 30 animals. There is a 10% adjustment accounted for potential sample exclusions during the course of the experiment.

The study used 30 male Wistar rats (*Rattus norvegicus*), aged two to three months with an average weight of 200-250 grams. These rats were in good health, had not undergone any prior research treatments, and were housed at the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara. A seven-day acclimatization period prior to the treatment was provided to ensure the rats' adaptation to their environment, during which they had unrestricted access to food and water. Rats with pre-existing abnormalities or those that expired before the conclusion of the experiment were excluded from the study. No replacements were made for deceased rats, provided the minimum sample size was maintained.

The rats were randomly assigned into six groups through a simple random sampling method by the laboratory assistants. Groups I to III received 3% Binahong leaf extract nanogel, while groups IV to VI were administered nanogel base as a negative control. The treatments were administered over a 28-day period, as this duration indicates the proliferation phase in socket healing and allows the observation of osteoblast differentiation from MSC.[4,7] All researchers, except those responsible for applying the gel to socket wounds, were blinded to the allocation of rats into groups.[7]

Experiment protocol

Wistar rats underwent intraperitoneal anaesthesia using a combination of ketamine 91 mg/kgBW (Agrovet Market, Peru) and xylazine 9.1 mg/kgBW (Interchemie, Netherlands) at a dose of 0.1 mL/100 g rat weight to mitigate procedural pain.[22] Subsequently, the left mandibular incisor was extracted using a luxation movement with an artery clamp (Wells Spencer, London).[23] Following extraction, the socket was rinsed with distilled water to remove debris.[7] The extraction procedure was performed by the same blinded veterinarian to ensure consistency across all groups.

The socket wounds in groups I to III were administered with 0.1mL of 3% Binahong leaf extract nanogel, while groups IV to VI were given 0.1mL of nanogel base. Administration of nanogel was done using a 1mL syringe (One Med Health Care, Indonesia) with no. 18 *abbocath* irrigation tip (PT. Terumo Indonesia, Indonesia) covering and contact directly with the entire socket wounds.[7] The gel applications were conducted twice daily: in the morning (8:00 a.m. to 10:00 a.m.) and in the afternoon (4:00 p.m. to 6:00 p.m.), for 28 days. This specific timing was selected to maintain a consistent schedule for gel application each day. A researcher, aware of the group allocation, performed the applications.[7]

Immunohistochemical analysis for BMP-2 expression

On the 7th (group I and IV), 14th (group II and V), and 28th days (group III and VI) post-extraction, rats from respective groups were euthanized with cervical dislocation for rapid and contamination-free tissue collection. The mandibles were removed, and socket wound tissue was excised. Tissue samples were fixed in 10% Buffered Neutral Formalin (BNF) solution (pH 6.8-7.0) at a ratio of 1:10 for 12-48 hours, followed by 10 days of immersion in 10% EDTA solution for decalcification. Tissue sections, 4 mm thick, were cut with a scalpel, placed in tissue cassettes, and processed in an automatic processor machine for dehydration. Subsequently, tissue embedding was carried out by placing the tissue in moulds submerged in liquid paraffin.[24,25]

Immunohistochemical staining was employed to evaluate the BMP-2 expression levels. The staining process followed a two-step technique using The EnVision+ Dual Link System kit. A Rabbit Polyclonal–anti-BMP-2 antibody was utilized as the primary antibody at a dilution of 1:100. Initially, paraffin

blocks were thinly sliced (4 μm thick) using a microtome, and the sections were mounted onto glass slides and soaked in warm water. Subsequently, the slides were placed on a hot plate (50-60°C) to soften the paraffin. After drying, the tissue sections were ready for staining. For immunohistochemical staining, coated glass slides with poly-L-lysine or silanized slides were employed to ensure tissue adhesion during the staining process. Standard preparation steps were followed, including deparaffinization, rehydration, and blocking of endogenous peroxidase activity. The tissue slides were then heated in a citrate buffer using a two-step microwave process, cooled, and rinsed with PBS (pH 7.4). Surrounding tissue areas on the slides were marked, and normal horse serum (5%) was applied, followed by a 5-minute incubation period.[26,27]

Primary antibodies (BMP antibodies) were applied at appropriate dilutions as per instructions, followed by a thorough cleaning with PBS (pH 7.4) and a tween. Secondary antibody application, conjugated with horseradish peroxidase (HRP), was performed for 30 minutes, followed by thorough cleaning. Counterstaining, dehydration, clearing, and mounting procedures were then carried out.[26,27] Then, observation under the microscope was conducted to assess the BMP-2 expression. The assessment of BMP-2 expression levels was conducted using the immunoreactive score (IRS), considering both the percentage distribution of stained cells and staining intensity. The final IRS was determined by multiplying the percentage distribution score by the staining intensity score, allowing for the classification of protein expression levels as mentioned in Fedchenko (Table 1).[28]

Table 1. Criteria for assessment of expression in immunohistochemical examination.

Parameter	Scoring	Interpretation
Cell distribution percentage	Score 0	No immunoreactive cells
	Score 1	Immunoreactive cells <10%
	Score 2	Immunoreactive cells 10%-50%
	Score 3	Immunoreactive cells 51%-80%
	Score 4	Immunoreactive cells >80%
Staining intensity	Score 0	No staining
	Score 1	Weak staining (pale)
	Score 2	Moderate staining (bright)
	Score 3	Strong staining (dark)
Final IRS	Score 0	No protein expression
	Score 1-3	Low protein expression
	Score 4-8	Moderate protein expression
	Score 9-12	High protein expression

Adapted from Fedchenko et al.[28]

Statistical analysis

The statistical analysis for BMP-2 expression, being categorical data, was analysed using the chi-square test. The significance level was set at p-value < 0.05. Statistical analysis was conducted using the statistical package for the social sciences (SPSS), version 22 (IBM Inc., USA), by a researcher who was aware of the group allocation.

3. Results

Immunohistochemical staining analysis

Immunohistochemical staining was employed to assess the expression levels of BMP-2 in the tissue samples obtained from the experimental groups. This analysis aimed to elucidate the distribution and intensity of BMP-2 staining, providing insights into its potential role in alveolar bone healing. Three key parameters were evaluated: the percentage distribution score, staining intensity score, and immunoreactive score (IRS).

The percentage distribution score reflects the proportion of cells exhibiting positive staining for BMP-2 within the tissue samples. Table 2 presents the distribution of staining intensity categories observed within each group.

Table 2. Percentage distribution score of BMP-2 expression in all groups.

Day	Group	Cell distribution				
		None	<10%	10-50%	51-80%	>80%
		n (%)	n (%)	n (%)	n (%)	n (%)
7	I	2(40)	3(60)	0	0	0
	IV	1(25)	3(75)	0	0	0
14	II	1(20)	3(60)	1(20)	0	0
	V	1(20)	4(80)	0	0	0
28	III	0	1(20)	3(60)	1(20)	0
	VI	1(20)	4(80)	0	0	0

The staining intensity score reflects the strength of BMP-2 staining observed in the tissue samples. Table 3 summarizes the distribution of staining intensity categories observed within each group.

Table 3. Staining intensity score of BMP-2 expression in all groups.

Day	Group	Staining Intensity			
		None	Weak	Moderate	Strong
		n (%)	n (%)	n (%)	n (%)
7	I	2(40)	3(60)	0	0
	IV	1(25)	3(75)	0	0
14	II	1(20)	4(80)	0	0
	V	1(20)	4(80)	0	0
28	III	0	5(100)	0	0
	VI	1(20)	4(80)	0	0

The immunoreactive score (IRS) combines the percentage distribution score and staining intensity score to provide an overall assessment of BMP-2 expression levels. It integrates both spatial and intensity aspects of BMP-2 staining, offering a comprehensive understanding of its expression dynamics. Table 4 summarizes the IRS values obtained for each experimental group. Microscopic images depicting BMP-2 expression in one sample of each group are presented in Figure 1 (expressions were pointed with an arrow).

Table 4. Staining intensity score of BMP-2 expression in all groups and chi-square test analysis.

Day	Group	Immunoreactive score				p value[a]
		None	Weak	Moderate	High	
		n (%)	n (%)	n (%)	n (%)	
7	I	2(40)	3(60)	0	0	0.635
	IV	1(25)	3(75)	0	0	
14	II	1(20)	4(80)	0	0	1.000
	V	1(20)	4(80)	0	0	
28	III	0	5(100)	0	0	0.292
	VI	1(20)	4(80)	0	0	

[a]chi-square test, significance at $p<0.05$.

These findings shed light on the distribution, intensity, and overall expression levels of BMP-2 within the alveolar bone healing process across different experimental conditions.

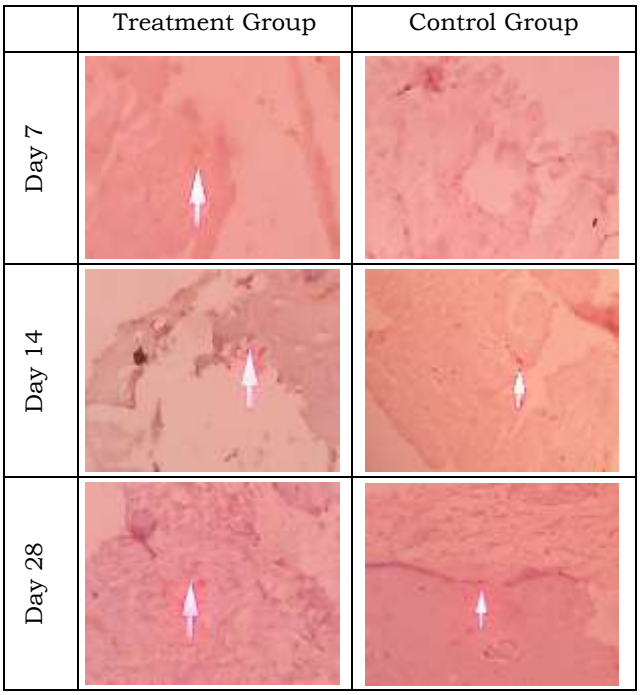

Figure 1. Microscopic analysis of BMP-2 expression in both experimental groups.

Statistical analysis

The statistical analysis was conducted using the chi-square test. The analysis aimed to assess the significant differences in BMP-2 expression levels among the experimental groups at various observation periods (7, 14, and 28 days). However, results indicated no statistically significant increase in BMP-2 expression among groups across all observation periods ($p>0.05$).

4. Discussion

One prevalent issue in dentistry is the occurrence of inadequate bone volume following tooth loss. This phenomenon occurs due to the rapid resorption of alveolar bone, which happens when there is no intraosseous stimulation from the periodontal ligament fibres.[29] Previous studies highlighted the crucial role of BMP-2 in osteogenesis.[3,4,30] and the potential of wound healing activity from the Binahong leaf extract on soft and hard tissues, observed in molecular[12,20,31,32], cellular[7,12–14,20,33] and clinical aspects.[7,34,35] However, there is still potential to fill the gap in advancing drug delivery systems to enhance therapeutic efficacy. Therefore, this investigation was designed to fill the gap by providing insights into the potential effect of Binahong leaf extract nanogel on BMP-2 expression level during the alveolar bone healing process.

The results showed an increase in cell distribution percentage (Table 2) and staining intensity (Table 3) in the treatment groups, although the final IRS did not imply statistical significance (Table 4). These findings indicate a potential influence of this novel approach on BMP-2 expression, which may implicate enhancing alveolar bone healing. Besides observing the molecular activity, we also found one subject from group IV died before its observation periods and two subjects were observed to develop abscesses in the extraction area to the base of the mandible, respectively in group IV and V. This sub-finding may indicate Binahong leaf extract nanogel did play an important role in the early inflammation phase and showed its antibacterial effect.

BMP-2 plays a crucial role in bone formation, particularly in the process known as intramembranous ossification.[3,12,36] This process is fundamental in the formation of bones like the mandible. MSCs in the repair area differentiate into osteoprogenitor cells, which then develop into osteoblasts. These osteoblasts secrete essential

components of bone tissue, leading to the formation of bone matrix. As osteoblasts proliferate and bind to the bone matrix, it undergoes calcification, contributing to bone tissue formation.[4,8,12,37] Additionally, in the inflammatory microenvironment protein expressions of BMP-2 in osteoblasts were found significantly decreased.[1] This theory highlighted the elimination of factors that could be prolonging the inflammatory period is very crucial.

Several studies explore the secondary metabolites present in Binahong leaf extract; including flavonoids, saponins, tannins, alkaloids, triterpenoids, steroids, glycosides, and phenolics; which contributed to the healing process through various mechanisms and activities.[9–11] Icariin, a flavonoid glucoside, enhances cell growth and formation of bone by increasing key genes following BMP-2 stimulation.[30] Anti-inflammatory properties of flavonoids (icariin, vitexin, apigenin, etc.) reduce capillary permeability and edema by inhibiting arachidonic acid pathways, including lipoxygenase and cyclooxygenase. This suppresses prostaglandins and leukotrienes biosynthesis, shortening the inflammation phase and accelerating the transition to the proliferation phase of bone healing. Additionally, flavonoids inhibit neutrophil degranulation and prevent leukocyte accumulation, thereby minimizing the inflammatory response and facilitating bone healing.[7,37–39]

The previous study showed that total saponins from Radix dipsaci could enhance the expression levels of BMP-2. This indicates that the activation of p38 and ERK (extracellular signal-regulated kinase) pathways likely plays a crucial part in elevating BMP-2 expression and promoting osteoblasts (MC3T3-E1) cells under saponin stimulation.[8] Saponin prevents infection by disrupting the pro-inflammatory cycle, thereby inhibiting bacteria from proliferating.[7,37] Other than saponins, alkaloids and tannins also known as antimicrobial agents disrupt bacterial nucleic acid synthesis and enzyme activity, halting metabolism and preventing infection from progressing.[37,40,41] Tannin also acts as an antioxidant, essential for counteracting the harmful effects of free radicals generated during wound healing. These free radicals have the potential to harm cell proteins, hindering their structure and impeding cell proliferation.[7]

Triterpenoid suppresses bacteria growth by disrupting the process of forming bacterial cell membranes.[37] Triterpenes found in Binahong leaf extract, oleanolic, and ursolic acid, also act as antiseptic, antioxidant, and anti-inflammatory agents that help in wound healing.[7] Polyphenols, including those found in Binahong leaf extract, exhibit various biological effects that are known to contribute to bone health. These compounds mitigate oxidative stress, reducing inflammation through proinflammatory signalling suppression, and regulating osteoblastogenesis, thereby exerting osteoimmunological effects.[42] Binahong leaf extract contains steroids and has demonstrated effectiveness in inflammation control, there's a suggestion of potential benefits beyond oral mucosal healing. The observed efficacy in inflammation control in recurrent aphthous stomatitis and potentially shorter healing periods hints at its plausible role in accelerating bone regeneration.[43]

Nanogel was chosen due to its advantageous properties, such as enhanced biocompatibility, permeability, stability, and controlled and targeted drug release. The capacity of nanogels to dissolve hydrophobic medications enhances their application in administering various oral treatments. Adjusting nanogel characteristics, influenced by factors such as temperature, cross-linking density, and surfactant concentration, provides avenues for adapting formulations to specific requirements in oral healthcare.[6,15,17] This suggests that the nanogel may have facilitated a more efficient and targeted delivery of active compounds to the site of action, potentially contributing to the observed improvements in cell distribution and staining intensity.

While direct evidence linking Binahong leaf extract to bone healing, particularly through BMP-2 modulation may not have been extensively studied, our findings contributed to the growing body of literature exploring natural compounds of Binahong

leaf extract and the advantages properties of nanogel for enhancing bone healing. Nevertheless, this study has several limitations, including a small sample size and short study duration. Additionally, the choice of outcome measures and assessment methods may have limitations in capturing subtle changes in BMP-2 expression.

Our study lays the groundwork for further research endeavours exploring the therapeutic potential of Binahong leaf extract nanogel in bone regeneration. Further investigations with larger sample sizes, longer study durations, more comparative groups, and more sensitive analytical techniques are warranted to confirm and build upon our findings. Furthermore, studies evaluating the underlying mechanisms of BMP-2 modulation by this novel approach and its clinical implications in dental practice are needed to fully employ its therapeutic benefits.

5. Conclusion

In conclusion, while the results of this research did not achieve statistical significance, they indicate a trend towards increasing efficacy in the application of 3% Binahong leaf extract nanogel concerning cell distribution percentage and staining intensity compared to the control group. Additionally, we found only in the control group did abscesses develop resulting in the premature death of one subject.

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