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Original Article

EFFECTIVENESS OF CHITOSAN FILM ON NEUTROPHILS AND MACROPHAGES COUNTS IN THE HEALING OF GINGIVAL INSICION IN WISTAR RATS (*RATTUS NORVEGICUS*)

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ABSTRACT

Objective: Incisional wounds caused by surgical interventions often result in complications, including infections. Chitosan is a promising material to promote healing. This study aimed is to determine the effectiveness of chitosan film as a periodontal dressing on the number of neutrophils and macrophages in chitosan film.

Methods: This *in vivo* experimental study used 36 rats with 3 mm gingival incisions. Rats were divided into three groups: chitosan film (treatment), Ora-Aid (positive control), and placebo (negative control). Dressings were applied twice daily for seven days. Rats were euthanized on days 1, 3, and 7 for neutrophil analysis, and on days 3, 7, and 14 for macrophage evaluation.

Results: The study showed that on day one, neutrophil counts were highest in the chitosan group, followed by the positive control, and lowest in the placebo group, with a significant difference ($p<0.05$). Neutrophil numbers decreased in all groups over time. By days 3 and 7, there was no significant difference between the Ora-Aid and chitosan groups ($p>0.05$). On day 3, macrophage counts were highest in the chitosan film group, followed by Ora-Aid and placebo, with a significant difference ($p<0.05$). By day 7, no significant difference was found between the chitosan and positive control groups ($p>0.05$).

Conclusion: Chitosan film demonstrates a higher number of neutrophils and macrophages during the early stages of healing compared to placebo. Therefore, chitosan film may be considered a potential wound dressing material to accelerate the healing process.

Keywords: Chitosan film, Chitosan, Neutrophils, Macrophage, Wound healing

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INTRODUCTION

Periodontal treatment often involves surgical procedures that result in incision wounds due to cuts on the oral mucosal tissue, such as gingivectomy, gingivoplasty, and flap surgery [1]. Therefore, in surgical procedures, it is important to cover the wound with a dressing to prevent contamination and minimize the risk of infection [2]. Periodontal packs are a form of periodontal dressing used to protect tissue from microorganism infection, control bleeding, and protect tissue from contact with food or the tongue when chewing or speaking, which can cause pain [3]. Long-term use of periodontal packs can increase cytotoxic, inflammatory, and tissue-irritating effects. While periodontal packs can protect wounds from mechanical trauma and maintain tissue stability, they do not accelerate wound-healing because they lack healing factors. All periodontal packs have the drawback of poor adhesion properties, which can lead to plaque accumulation and slow the healing process [4].

Film formulations can be an alternative choice for periodontal dressings, offering a more aesthetic and comfortable option. Film as wound dressing is also easier to apply to the tissue surface due to its good adhesive and flexible properties, which can protect the wound surface, reduce pain, and control bleeding [5]. Min *et al.*'s research found that using wound dressing film in flap surgery patients can reduce bleeding, pain, and discomfort while eating after surgery. The use of this dressing also did not show any hypersensitivity reactions [6]. Another study conducted by Kang *et al.* on the effectiveness of wound dressing film on intraoral wounds in rabbits found that this formulation can improve the wound closure process by increasing collagen accumulation during the wound healing process [5].

Nowadays, using natural materials as medicine is widely looked at and developed, one of which is chitosan. Chitosan is a deacetylated chitin-based derivative found in the exoskeleton waste of marine animals such as shrimp, crab, or lobster, which is usually a by-product of the seafood industry [7, 8]. Chitosan is proven to be effective in accelerating wound healing because it has mucoadhesive, biocompatible, antimicrobial, non-toxic properties and biodegradable polymer that can be easily engineered [10, 11]. Chitosan is an effective drug carrier due to its high cellular transfer efficiency and enhances drug absorption by prolonging the contact time between the drug and the cell membrane. Its suitability for various non-invasive administration routes, such as oral [12]. Chitosan can act as an anti-inflammatory agent and a promising drug delivery system, stimulating cell proliferation and remodeling [9, 10, 13]. It can also induce platelet adhesion and activation in the wound healing process for blood clotting and to activate polymorphonuclear (PMN) cells and inflammatory cells such as macrophages for hemostatic functions [14].

Neutrophils, known as polymorphonuclear leukocytes, are the most abundant leukocytes and are part of innate immunity [15]. In the early stages of wound healing, neutrophils are essential for phagocytosing pathogenic microorganisms and producing proteases [16]. Macrophages, as one of the inflammatory cells, play a crucial role as large mononuclear phagocytic cells that produce cytokines and growth factors, stimulating fibroblast proliferation, collagen production, and the formation of new blood vessels. They are key regulatory cells mediating the transition from the inflammatory to the proliferative phases [17].

This is consistent with research conducted by Sularsih *et al.*, which found that 1% chitosan gel can release the cytokine tumor necrosis alpha (TNF-α) to activate macrophage cells on the 3rd and 4th d, thereby accelerating the wound healing process [18]. Previous research by Weda *et al.*, demonstrated that administering chitosan gel at concentrations of 1.25% and 2.5% significantly accelerated wound healing. This study also showed that the optimal concentration of chitosan gel is 2.5%, providing the best results in burn wound healing in white rats by enhancing the activity and accumulation of polymorphonuclear cells [19].

Based on the description, the researchers are interested in determining the effectiveness of applying a chitosan film as a periodontal dressing by evaluating the number of neutrophils and macrophages in accelerating the wound healing process following gingival incisions in Wistar rats (*Rattus norvegicus*).

MATERIALS AND METHODS

This study is an *in vivo* laboratory experimental research with a post-test control group design. The Research Ethics Committee has approved this research for Animal Studies of the Universitas Sumatera Utara with registration number 0799/KEPH-FMIPA/2023). The sample consists of 36 male Wistar rats aged 2-3 mo, weighing 180-200 g, and in healthy condition. The samples are divided into three groups: the treatment group given chitosan film, the positive control group given Ora-Aid® (Surgical Smart TM, TBM Corp., Canada) and the negative control group given a Film without active ingredients. The sample size calculation in this study used the Federer formula (□−1)(t−1) ≥ 15, where □ represents the number of samples per group and t represents the number of groups.

Based on this formula, the required number of wistar rats per group is four, with observations conducted on different days—day 3, day 7, and day 14.

The preparation of chitosan film as periodontal dressing

250 mg of chitosan powder is dissolved in 3% acetic acid, poured into a beaker glass, and stirred with 500 RPM for 24 h using a magnetic stirrer bar at room temperature until homogeneous. Once homogeneous, the pH of the chitosan is checked using a pH meter. Add 0.2 N NaOH drop by drop if the pH of the chitosan falls below the normal range. 50 mg of PVP is dissolved in distilled water, covered with aluminum foil, and left to stand for 24 h. Afterward, Sodium Carboxymethyl Cellulose (CMC-Na) is dissolved in hot water for 20 min. The chitosan, Polivinilpirolidon (PVP), and CMC-Na are combined with the addition of 15 ml of polysorbate to integrate the three polymers. The mixture is placed in a petri dish at room temperature (20 °C-25 °C) for 30 min to remove any trapped air bubbles. The petri dish is then dried in an oven at 40 °C-42 °C for 2-3 d to obtain the film preparation. Once dry, the film is cut into 5 mm x 2 mm pieces using a blade.

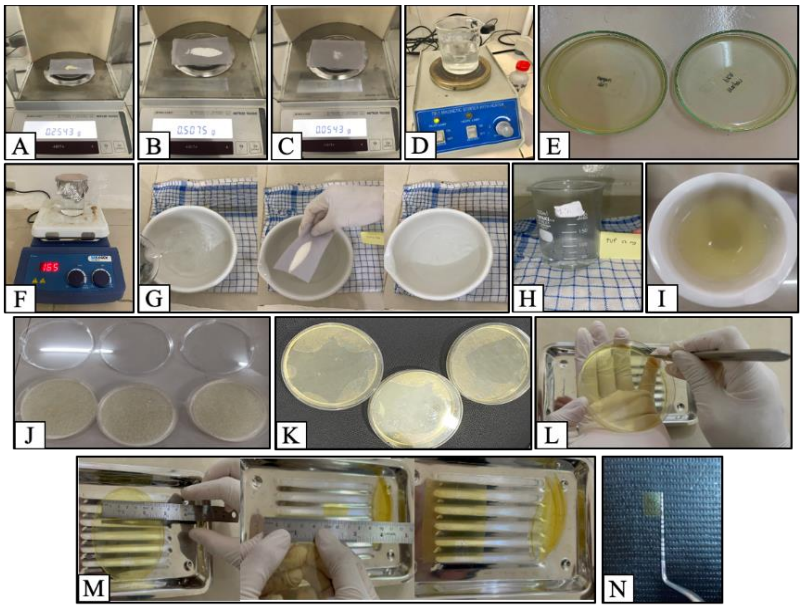

Fig. 1: Preparation of Chitosan Film (A) Chitosan 250 mg (B) CMC-Na 500 mg (C) PVP 50 mg (D) Dissolution of chitosan (E) Chitosan solution (F)Heating of distilled water (G)Expansion of CMC-Na (H)Dissolution of PVP (I)Combination of Chitosan+CMC-Na+PVP+Polysorbate (J) Preparation of chitosan film in a petri dish (K) Dried chitosan film (L)Removal of film from the petri dish (M) Measurement and cutting of chitosan film (N) Chitosan film cut to 5 mm x 2 mm

The preparation of film without active ingredients as placebo

500 mg of CMC-Na was dissolved in hot water for 20 min. Then, 50 mg of PVP was dissolved in distilled water, covered with aluminum foil, and left to stand for 24 h. The two polymers were then combined in a mortar and homogenized. Once homogeneous, the polymer mixture was poured into a petri dish at room temperature (20 °C–25 °C) to remove trapped air bubbles and was dried for approximately 1–2 d to form a film preparation. After drying, the film was cut into 5 mm × 2 mm pieces using a blade [20].

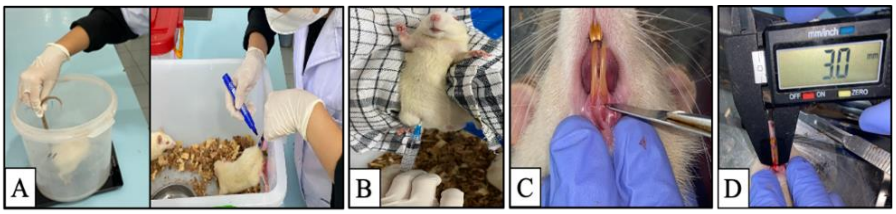

Fig. 2: Applying chitosan film post-gingival incision in rats (A) Weighing and marking the rats (B) Intraperitoneal anesthesia (C) Labial gingival incision of the lower central incisor (D) 3 mm gingival incision

Application of film as periodontal dressing to post-incision wounds in rats

The rats were anesthetized with an intraperitoneal injection of a ketamine and xylazine mixture. A horizontal incision was made on the labial gingival surface of the lower central incisors, extending 3 mm to the bone in one stroke using a sterile no. 15 stainless steel surgical blade, which was replaced after every three incisions.

Immediately after the incision, the chitosan film was applied to the wound area twice daily for seven days, in the morning and evening. The rats were euthanized on days one, three, and seven to examine the histology for neutrophil cell counts and on days one, seven, and fourteen to examine macrophage and fibroblast cell counts.

Procedure for obtaining the mandible and histological sample preparation process

The test animals are euthanized by cervical dislocation. The deceased rats are placed on a container. The neck is separated from the head using a scalpel and blade. The mandible is then separated from the head using a blade and surgical scissors until an intact mandible segment is obtained. The lower jaw segment is dissected, removed, and washed with running water to clean the blood and bacterial contamination sample. The mandible segment is then placed in a container with 10% Neutral Buffer Formalin to preserve the integrity of the sample for 24 h. The remaining parts of the rat's body are buried following animal ethics.

The preparation of histological specimens of mandibular segments involved decalcification with 2% EDTA, fixation in 10% buffered formalin, infiltration with liquid paraffin, embedding, sectioning, and staining with *haematoxiline-eosin.*

Deparaffinization was performed using xylol three times, each for 5 min, to dissolve the paraffin in the tissue. Rehydration was carried out by immersing the object glass in 100%, 96%, and 80% alcohol, each for 5 min, followed by rinsing under running water for 5 min. The object glass was then immersed in hematoxylin stain for 5 min, followed by another 5 min rinse. Next, the object glass was dipped in 1% acid alcohol 1–2 times and rinsed again for 5 min. The object glass was then immersed in 1% eosin stain for 2–3 min. Dehydration was performed by immersing the object glass in 80%, 96%, and 100% alcohol, each for 5 min. Mounting was then performed using Canada balsam or Entellan to preserve the stained tissue, followed by covering with a cover glass and labeling [21].

Histological observation procedure

Research data were obtained by observing the histological preparations using a binocular microscope at 400× magnification, across five fields of view, aided by image processing software. Cell counting was conducted systematically for each preparation, starting from the top-left corner, moving right, then upward, and so on, ensuring complete coverage of the field of view. The average number of cells per sample was calculated by determining the mean cell count across the five fields of view.

RESULTS

Evaluation of the characteristics of chitosan film as periodontal dressing

Organoleptic test

Organoleptic testing was performed by visually evaluating the color, texture, and transparency of the chitosan film [22-24].

Thickness test

The thickness of the chitosan film is measured using a digital micrometer caliper at five different points: the top, bottom, right, left, and center. This process is repeated five times, and the average thickness is calculated [23-25].

Table 1: Organoleptic observation results of chitosan film and placebo

Criteria	Formulation	Preservation time				
		Day 1	Day 7	Day 14	Day 21	Day 28
Color	Chitosan Film	Yellow	Yellow	Yellow	Yellow	Yellow
	Placebo	Clear	Clear	Clear	Clear	Clear
Flexibility	Chitosan Film	Flexible	Flexible	Flexible	Flexible	Flexible
	Placebo	Flexible	Flexible	Flexible	Flexible	Flexible
Texture	Chitosan Film	Soft	Soft	Soft	Soft	Soft
	Placebo	Soft	Soft	Soft	Soft	Soft
Homogeneity	Chitosan Film	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
	Placebo	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous

Table 2: Results of the thickness test for chitosan film and placebo

	Thickness				
Formulation	Upper left point (mm)	Upper right point (mm)	Lower right point (mm)	Lower left point (mm)	Middle point (mm)
Chitosan film	1:5	1:4	1:5	1:4	1:4
Placebo	1:1	1:1	1:3	1:1	1:2

Table 3: The differences in neutrophil counts on days 1, 3, and 7 post-incision across all groups

Group	Mean neutrophil counts ($\bar{x}$±SD)			
	Chitosan film	Ora-aid	Placebo	P-value
Day-1	102.33±32.68	194.53±5.62	21.00±1.05	.000*
Day-3	28.33±3.52	32.33±1.67	15.93±0.50	.000*
Day-7	5.06±0.23	5.80±0.22	0.20±16.26	.008*

*One-Way ANOVA Statistical Test, significant differences among groups ($p<0.05$)

Table 4: The comparison of neutrophil counts between groups on Days 1, 3, and 7 post-incision

Group		p-value		
		Day-1	Day-3	Day-7
Chitosan Film	Ora-Aid	.001*	.074	.784
Ora-Aid	Placebo	.000*	.000*	.006*
Placebo	Chitosan Film	.002*	.001*	.005*

*Post Hoc LSD Test, significant difference between two group ($p<0.05$)

Based on the results of Hematoxylin-eosin staining of gingival tissue post-incision, it was observed that the number of neutrophils, appearing as oval or round-shaped cells with bluish-red color, was lower in the negative control group compared to the treatment and positive control groups. The highest number of neutrophils was observed on the first day after incision in the positive control group.

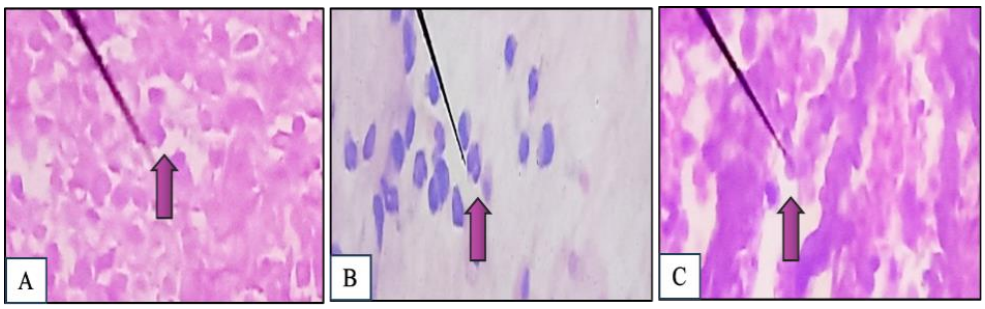

Fig. 3: Histological appearance of neutrophil cells on day 1 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

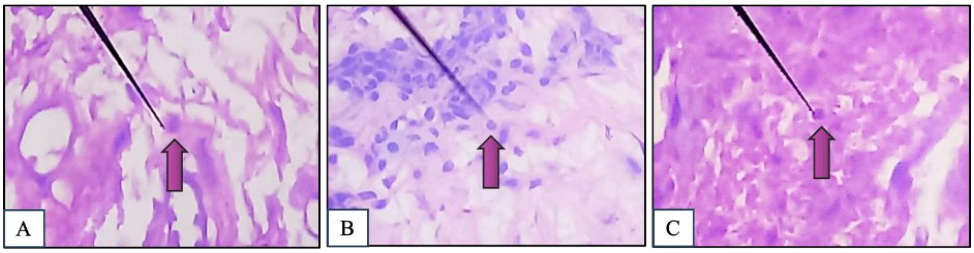

Fig. 4: Histological appearance of neutrophil cells on day 3 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

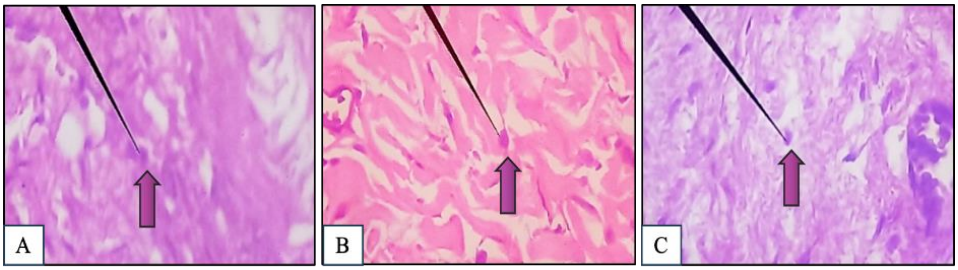

Fig. 5: Histological appearance of neutrophil cells on day 7 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

Table 5: The differences in macrophage count on days 3, 7, and 14 post-incision across all groups

Group	Mean macrophage counts (x̄±SD)			
	Chitosan film	Ora-aid	Placebo	p-value
Day-3	8.93±0.115	6.86±0.757	3.20±0.800	0.027*
Day-7	5.20±1.216	2.00±0.721	8.00±2.433	0.012*
Day-14	1.66±0.115	1.13±0.115	2.40±0.200	0.026*

Kruskal-Wallis test on Day-3 and day-14, One-Way ANOVA test on Day-7, *p<0.05 = significant difference between groups

Table 6: The comparison of macrophage count between groups on days 3, 7, and 14

Group		p-value		
		Day-3	Day-7	Day-14
Chitosan Film	Placebo	0.046*	.079	0.046*
Ora-Aid	Chitosan Film	0.046*	0.052	0.043*
Placebo	Ora-Aid	0.050*	.004*	0.046*

Mann-Whitney test on Day-3 and day-14, Post Hoc LSD test on Day-7, *p<0.05 = significant difference between groups

Histological observations on day three post-treatment revealed that macrophage cells were more abundant in the group treated with chitosan film compared to the group treated with Ora-Aid, and least abundant in the placebo group. On day seven, histological findings indicated that macrophage cells remained more numerous in the treatment and positive control groups,

while their numbers were lower in the negative control group, although higher than on day three. By day fourteen, histological observations showed a decrease in macrophage cells across all treatment groups.

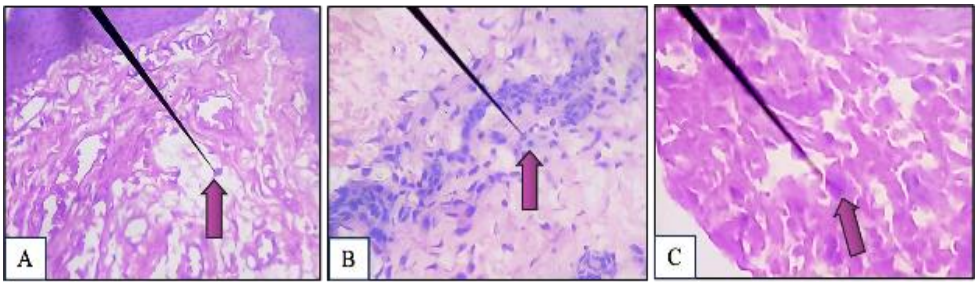

Fig. 6: Histological appearance of macrophage cells on day 3 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

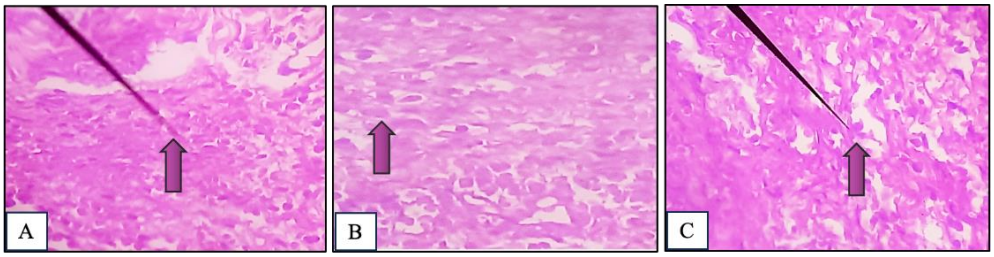

Fig. 7: Histological appearance of macrophage cells on day 7 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

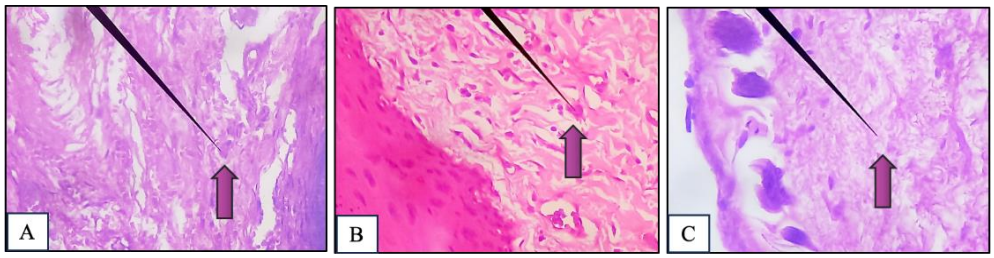

Fig. 8: Histological appearance of macrophage cells on day 14 with 400 times magnification after treatment with (A) Chitosan Film (B) Ora-Aid (C) Placebo

DISCUSSION

The chitosan film formulation creates an ideal moist environment at the wound site, which reduces the risk of dehydration and promotes faster wound closure. This aligns with findings by Colobatiu *et al.*, who noted that chitosan film is easy to apply and optimally provides a moist environment for wounds, hydrating the area by absorbing exudate. Their research also revealed that chitosan film can stimulate and accelerate the wound-healing process [26]. Similarly, Seyed *et al.* demonstrated that chitosan film effectively controls inflammation, further promoting wound healing [27]. Moreover, the presence of 2.5% chitosan content in the film enhances its ability to accelerate the healing process, as seen in the study by Weka *et al.*, which showed that 2.5% chitosan gel optimally speeds up burn wound healing [19]. Additionally, Pereira *et al.*'s research supports the effectiveness of chitosan film in stimulating wound repair [28].

The simple form of chitosan film allows for ease of application. The solvent casting method used in the preparation of chitosan film is also considered cost-effective, making it suitable for production in resource-limited settings. This method enables the fabrication of films with adjustable thickness and mechanical properties, allowing for controlled release of active substances [29]. However, the dynamic conditions of the oral environment, such as the presence of saliva and tongue movement, can affect the mucoadhesion of chitosan films. Therefore, formulations that can maintain adhesion under such conditions are necessary to ensure.

In this study, both the treatment group and the positive control group showed signs of healing, whereas one rat in the negative control group developed an abscess on a certain day.

The administration of a chitosan film as a periodontal dressing to gingival incision wounds in rats resulted the number of neutrophils was higher than in the negative control group. This showed chitosan's role in accelerating the migration of inflammatory cells such as neutrophils and enhancing the granulation phase, thereby expediting the wound healing process [32].

Neutrophil infiltration occurs during the inflammatory phase, typically within 72 h after the wound occurs, playing a crucial role in removing debris and preventing infection by phagocytosing pathogenic microorganisms and producing proteases. While neutrophils are essential for the host defense system, their prolonged presence at the wound site can disrupt normal wound healing and lead to chronic wounds. Thus, shortening the inflammatory phase is critical for accelerating the wound healing process [33, 34]. During the initial phase of wound healing, chitosan facilitates the infiltration and migration of polymorphonuclear neutrophils (PMNs) and promotes granulation by inducing dermal

fibroblast proliferation. This process helps cleanse the wound of foreign materials through neutrophil activity. Additionally, chitosan induces platelet adhesion and activation, supporting rapid blood clot formation during the hemostasis phase [33-35].

Histological analysis revealed that the highest neutrophil count occurred on the first day post-incision. During the early phase of wound healing, chitosan enhances the infiltration and migration of PMNs and stimulates granulation through dermal fibroblast proliferation. This process underscores the role of neutrophils in clearing foreign materials from the wound and promoting the progression of healing [35, 36]. The study by Syafruddin *et al.* showed the effectiveness of chitosan gel and gentamicin ointment in wound healing on white rats with incision wounds. Their study found a higher leukocyte count on day three in the chitosan ointment group compared to the control group treated with gentamicin ointment, demonstrating chitosan's effectiveness in accelerating the wound healing process [32]. Neutrophil count decrease from day three to day seven post-incision in all groups, with the chitosan film treatment group showing a significant decrease than the placebo group. By days 3 and 7, there was no significant difference between chitosan groups and the positive control ($p>0.05$). The research by Suharto *et al.* noted that neutrophil numbers peak during the inflammatory phase and subsequently decrease through apoptosis as wound healing progresses. Prolonged neutrophil presence beyond day one can delay epithelial repair and impede wound healing [38].

Additionally, this study supports findings by Guan *et al.*, which describe chitosan's dual action during the inflammatory phase: inhibiting arachidonic acid metabolism and enhancing macrophage activity. These processes reduce inflammation, as evidenced by a decline in neutrophil counts, highlighting the importance of shortening the inflammatory phase for effective wound healing [39].

The findings are also consistent with observations by Leoni *et al.*, who stated that neutrophils infiltrate the wound area within the first 12 h and are subsequently phagocytosed by macrophages or fibroblasts by day three. Prolonged neutrophil presence can cause tissue damage, extended inflammation, and chronic wounds. While neutrophils play a critical role in phagocytosing pathogens, the proteases and antimicrobial substances they release are nonspecific and can harm surrounding tissues. This underscores the necessity of timely resolution of neutrophil activity to avoid hindering the healing process [40].

The number of macrophages on the third day differed significantly among the groups ($p<0.05$), and were highest in the chitosan film group, followed by positive control and placebo. The peak in macrophage numbers on day three plays a critical role in accelerating the wound healing process, as macrophages are the dominant cell type during the late inflammatory phase [41, 42]. Macrophages act as phagocytic cells, removing debris and pathogens, and are crucial for releasing growth factors that stimulate fibroblast proliferation and activation, which are key to tissue repair [43, 44]. The number of macrophages decreases as the healing process gradually progresses, which begins to decline slowly from day 3 to day 14, as the process enters the proliferative phase. By day 7, no significant difference was found between the chitosan and positive control groups ($p>0.05$). During the proliferative phase, macrophages release growth factors such as TGF-β, which function in the chemotaxis of fibroblasts [44].

In this study, administering chitosan film showed number of macrophages is the highest compared to positive control and the placebo group. These findings are consistent with the study by Sularsih *et al.*, which showed that applying 1% chitosan gel releases the cytokine TNF-α to activate macrophages by day three, thereby expediting wound healing [18]. Similarly, research by Adistya *et al.* found that applying 3% chitosan gel also increases macrophage counts, further enhancing the healing process [45].

Chitosan's efficacy in wound healing is attributed to its active compound, N-acetylglucosamine, which promotes PMN migration and induces macrophage activity by stimulating chemotaxis and releasing growth factors like PDGF and TGF-β. The study by Puspita *et al.*, reported that the N-acetylglucosamine monomer binds to macrophages' primary mannose receptor, initiating internalization, migration, and proliferation of these cells [46]. Macrophage activation enhances metabolic activity, secretion of growth factors, and production of angiopeptin, which promotes angiogenesis, improves blood supply, and facilitates cell regeneration. These processes collectively accelerate the wound healing process [47].

On days seven and fourteen, the number of macrophages decreased as the healing process progressed. This decline aligns with the wound entering the proliferation phase, characterized by a reduction in inflammatory cells and their replacement by fibroblast proliferation. Groups treated with chitosan film and positive control exhibited a significant decrease in macrophage numbers, with the most notable reduction observed in the positive control group. In contrast, the placebo group showed a higher macrophage count on day seven compared to the groups treated with chitosan film and positive control, indicating that the inflammatory process in the placebo group was still active, with macrophages continuing to dominate.

The relatively high macrophage count in the placebo group on day seven suggests ongoing inflammatory activity and potential infection. This aligns with the observation of suppuration at the incision site in the placebo samples. In contrast, no signs of infection were observed in the chitosan film group. This can be attributed to chitosan's well-documented antibacterial properties. Chitosan contains lysozyme, aminopolysaccharide groups, amine groups, and polycationic compounds, all of which contribute to bacterial cell death [10, 48, 45]. Its antimicrobial activity disrupts bacterial cell membranes by increasing the permeability of both the inner and outer membranes, causing cell lysis in g-positive and g-negative bacteria alike [40]. Additionally, the antimicrobial properties of chitosan not only prevent infection but also promote the repair of damaged tissue, ensuring a smoother transition from the inflammatory phase to the proliferation phase of wound healing [48-50].

CONCLUSION

The administration of chitosan film as periodontal dressing effectively accelerates the healing process following a gingival incision. Chitosan film is a promising future alternative for periodontal dressings, as it possesses bioactive properties along with healing, antibacterial, and anti-inflammatory effects. To improve its therapeutic efficacy, the formulation should be further optimized, for instance by incorporating bioadhesive agents to enhance mucosal adhesion. Such advancements would not only facilitate broader clinical applicability but also ensure the film's stability and functional performance within the dynamic conditions of the oral cavity.

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AUTHORSS CONTRIBUTIONS

Irma Ervina has generated the research plan, given guidance and revised the menuscript. Audry Tracy, Thania Azarya, and Vanny Anastasya have conceptualization, methodology, data collection, and revised the manuscript. Denny Satria, Pitu Wulandari, Armia Syahputra, Aini Hariyani N, Rini Oktavia, and Zulkarnain, and Hesty Nurcahyanti have given guidance and supervision to carry out this study.

CONFLICT OF INTERESTS

Declared none

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