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The Effect of Nano Chitosan *Xylotrupes gideon* on Fibroblast Proliferation and Collagen Deposition in the Oral Mucosa of *Rattus norvegicus*

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Abstract

Lacerations of the oral mucosa are a common clinical problem and require an efficient healing process. The wound healing process involves the haemostasis, inflammation, proliferation, and maturation phases. During the proliferation phase, fibroblasts play an important role in migration and proliferation to produce collagen, which connects the wound's edges and provides strength and stability to the scar tissue that forms. Chitosan from the horned beetle (*Xylotruphes gideon*) contains active compounds that are thought to accelerate the healing process by increasing the activity of inflammatory cells and fibroblast proliferation. Physical modification into nanoparticles facilitates penetration into the mucosal layer, thereby accelerating collagen production and re-epithelialization. This study aimed to determine the effect of X. gideon nanokitosan administration on increasing fibroblast proliferation and collagen formation in vivo. The study was divided into five groups: positive control, negative control, and nano chitosan treatment at 3000, 1500, and 750µg/mL. All groups of experimental animals were previously given an injury to the right cheek mucosa with a No. 12 scalpel blade, and nano chitosan was administered topically. The increase in fibroblasts and collagen deposits was determined by HE and Masson's Trichrome staining on days 3, 7, and 14, which were observed microscopically and using ImageJ. The results showed a significant increase in fibroblasts on day 3 and collagen fibre deposition on day 14 in all treatment groups. The group with 750 ppm nano chitosan showed the best results in accelerating wound healing regarding fibroblast proliferation and collagen deposition. Conclusion: Nano chitosan *X. gideon* at a concentration of 750 ppm effectively accelerates wound healing in the cheek mucosa of mice by increasing fibroblast proliferation and collagen deposition.

Keywords: Collagen; Fibroblasts; Nano chitosan.

INTRODUCTION

A laceration is a type of wound that occurs due to trauma or direct contact with a sharp object that affects a part of the body (Susanto et al., 2023), one of which is the oral mucosa. Lacerations of the oral mucosa can be a common clinical problem due to its vital function in chewing, speaking, and swallowing (Eghbali et al., 2024). The presence of wounds in the oral cavity not only causes pain but also has the potential to trigger infections that prolong healing time (Eghbali et al., 2024). The wound will undergo a healing process involving complex, dynamic biological activities that play an important role in maintaining the integrity and function of body tissues after damage (Wilkinson & Hardman, 2023) (Wilkinson & Hardman, 2023). The wound healing process involves three main stages, namely the inflammatory, proliferation, and remodelling phases, in which fibroblasts and collagen deposits play a central role in tissue restoration. However, disruptions in this process will lead to complications such as infection and poor scarring (Wilkinson & Hardman, 2023).

Oral mucosal wound treatment usually involves topical agents such as hyaluronic acid and corticosteroids. However, the potential for side effects and allergies is a significant obstacle to the use of these synthetic drugs (Eghbali et al., 2024). Research on substances that can accelerate the wound healing process continues to develop. Natural ingredients are an alternative option for treating inflammation and accelerating wound closure, as they have milder side effects and are readily available (Susanto et al., 2023), one of which is biopolymer-based wound healing therapy, such as chitosan.

Chitosan is a natural polysaccharide produced by the deacetylation of chitin, which is derived from the exoskeletons of crustaceans such as shrimp and crabs, as well as insects. Chitosan has bioactive properties such as

biocompatibility, biodegradability, antimicrobial activity, and accelerating wound healing (Komariah et al., 2023). According to Novy et al (2025), the physical modification of chitosan into nanoparticles ranging from 10 to 1000 nm can increase its penetration into tissue and enhance its effectiveness. The nano size allows chitosan to penetrate the mucosal layer (Yusuf et al., 2023). One source of chitosan that is quite good but still underutilized is the horned beetle (*Xylotrupes gideon)*. This insect, commonly found in Southeast Asia, including Indonesia, has a chitosan content of around 48.32%, higher than conventional sources such as shrimp (26.2%) (Veronica et al., 2021).

Scratch wounds on the oral mucosa, particularly in *Rattus norvegicus* model rats, are often used in preclinical research due to their similarity to humans in terms of tissue physiology and regenerative capacity, making them an appropriate model for testing the efficacy of nano-chitosan *X. Gideon*. Previous studies have shown that a 400 µg/mL concentration of horned beetle nano-chitosan is capable of reducing the production of fibroblast ROS induced by stressors (Priscilla et al., 2023) and that chitosan encapsulating lemongrass leaf extract is capable of increasing fibroblast proliferation and migration (Andikoputri et al., 2021; Veronica et al., 2021). As there has been no research on using nano hornbeam chitosan to increase proliferation and collagen deposition, the researchers were interested in conducting this study to examine the potential of nano hornbeam chitosan in increasing proliferation and collagen deposition in healing incision wounds.

MATERIALS AND METHODS

Materials

The study was an in vivo laboratory experiment using *R. norvegicus* test animals that had passed an ethical review with number 0780/UN2.F1/ETIK/2019. The test animals were studied at the Laboratory of the Department of Nutrition, Faculty of Medicine, University of Indonesia. The research subjects were male Wistar strain white rats (*Rattus norvegicus*), aged 2-3 months, weighing 200-300 grams, which were kept per the UI nutrition science laboratory standards. The test animals were randomly divided into five groups of 5 rats each (25 rats in total). The groups consisted of a positive control group (0.2% hyaluronic acid, Aloclair®) (K+), a negative control group given distilled water (K-), and treatment groups given nano chitosan at concentrations of 3000 µg/mL (P1), 1500 µg/mL (P2), and 750 µg/mL (P3). Chitin from rhinoceros beetles (*X. gideon*) was sourced from Dramaga, Bogor, West Java, and underwent demineralisation, deproteinization, decolourization, and deacetylation processes to produce chitin with a deacetylation degree of 83% (Komariah et al., 2023; Komariah & Astuti, 2012).

Procedures

Nano Chitosan

Nano chitosan was produced using the ionic gelation method by weighing chitosan powder, dissolving it in 1-2% acetic acid, and adding distilled water up to 500 mL. The solution is stirred with *a magnetic stirrer (*IKA™ RH Basic 2, Germany) for two hours, with the first 30 minutes heated to 40ºC, then 0.1% sodium tripolyphosphate (Sigma-Aldrich®, USA) is added. A total of 40 mL is added drop by drop and stirred again for one hour. After that, add 0.1% Tween 80 (Merck®, France), 0.1 mL, and stir again for 30 minutes. The solution was then neutralized with 0.1 M NaOH (Merck®, Germany) to a neutral pH. The stock nano chitosan solution with a 3000 µg/mL concentration was diluted to test concentrations of 1500 and 750 µg/mL. This nano chitosan was ready for use and stored under sterile conditions for research.

Animal experimentation

Test animals that had been acclimatised for one week were anaesthetised using a combination of ketamine and xylazine, which was injected intraperitoneally (Komariah et al., 2018). A scratch wound was made on the right cheek mucosa using a size 4 x 3 mm number 12 scalpel blade. It was applied topically to the wounds in the P1, P2, and P3 treatment groups at concentrations appropriate to each group. The positive control group was treated with 0.2% hyaluronic acid, while the negative control group was only given placebo. The treatment was routinely carried out daily (Limay et al., 2019). Observations were made on days 1, 3, 10, and 14 after treatment (Cialdai et al., 2022a; Rognoni et al., 2018; Rusnedy et al., 2023; Zhou et al., 2013). At each observation time, one mouse from each group was sacrificed for right cheek mucosa tissue collection. The tissue was collected, washed using physiological saline, and fixed with 10% formalin solution for histological preparation.

Tissue Preparation and Staining

The tissue was subjected to dehydration, clearing, and infiltration using an *automatic tissue processor*. It was then embedded in paraffin blocks and sectioned at a thickness of 3–5 µm using a microtome. The tissue preparations were then stained with Haematoxylin-Eosin (HE) staining to count the number of fibroblasts and Masson's Trichrome staining to observe collagen fibre deposition.

Data Analysis

The number of fibroblasts and percentage of collagen deposits were analyzed using the ImageJ application based on microscopic observation. The data were statistically analyzed using SPSS version 23.0 with the Shapiro-Wilk normality test, followed by one-way analysis of variance (ANOVA) and Tukey's post hoc test

to determine significant differences between treatment groups. The significance level was $p < 0.05$ (Komariah et al., 2023).

RESULTS AND DISCUSSION

Macroscopic Observation of Wound Length

Observations on day 1 showed that the wound length in all treatment and control groups was relatively similar, with no significant differences ($p > 0.05$), with an average wound length of approximately 3.15–3.36 mm. On day 3, there was a significant decrease in wound length in the group given nano chitosan, especially the 750 ppm concentration group (P3), which showed wound shrinkage with an average of 1.93 mm, compared to the negative control (K-), which still had an average wound length of 2.64 mm. The results of wound observations in the study groups can be seen in Table 1.

Table 1. Wound measurement results in the study groups.

Group	N	Wound Measurement Results (mm) Day			
		1	3	10	14
K+	5	3.15 ± 0.16^{a}	2.25 ± 0.11^{bcd}	1.06 ± 0.04^{gh}	0
K-	5	3.18 ± 0.30^{a}	2.64 ± 0.30^{b}	2.04 ± 0.35^{cd}	0
P1	5	3.30 ± 0.26^{a}	2.43 ± 0.17^{bc}	1.59 ± 0.23^{ef}	0
P2	5	3.36 ± 0.16^{a}	2.11 ± 0.20^{cd}	1.41 ± 0.14^{fg}	0
P3	5	3.22 ± 0.60^{a}	1.93 ± 0.19^{de}	0.91 ± 0.10^{h}	0

[a-h] In the column, significant differences are indicated ($p < 0.05$). The lowercase letter "a" indicates a higher value compared to group "b", while "ab" indicates a non-significant difference ($p>0.05$).

On day 10, the difference in wound length was still significant, with the P3 group showing an average reduction of 0.91 mm, while the negative control group still had a longer wound of 2.04 mm. On day 14, all groups showed completely closed wounds with no visible macroscopic wound remnants. Wound observation on day 3 (Figure 1).

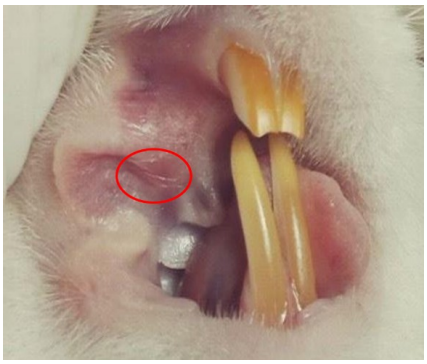


Figure 1. Results of wound observation on day 3 in the P3 treatment group.

Microscopic Observation of Fibroblast Count

Observation of the research results from the number of cheek mucosa fibroblasts in mice showed that on the first Day, the number of fibroblasts was relatively low and uniform in all groups, with group P1 showing the highest number of 72.2±1.48 cells/field of view compared to other groups, which ranged from 61.2 to 69.4 cells/field of view. On the third Day, there was a significant increase in the number of fibroblasts in the K+ group, namely 110.4±9.91 cells/field of view, indicating active proliferation stimulation, followed by groups P3 and P2 with 101.6±2.30 and 98.2±1.30, respectively. Meanwhile, the K-group increased but remained the lowest with a cell count of 83.0±0.71. The results of fibroblast counts observed on days 1, 3, 10, and 14 can be seen in Table 2.

Table 2. Results of fibroblast count calculations in the research group.

Group	Number	Number of Fibroblasts (cells) on Day			
		1	3	10	14
K+	5	65.6 ± 1.14^{jk}	110.4 ± 9.91^{a}	92.8 ± 1.79^{de}	59.6 ± 1.14
K-	5	61.2 ± 1.64^{k}	83.0 ± 0.71^{fgh}	81.4 ± 1.14^{gh}	79.6 ± 1.14^{h}
P1	5	72.2 ± 1.48^{i}	88.4 ± 1.14^{ef}	87.4 ± 1.52^{efg}	61.0 ± 1.22^{k}
P2	5	65.2 ± 1.92^{jk}	98.2 ± 1.30^{cd}	97.0 ± 0.71^{cd}	64.4 ± 1.34^{jk}
P3	5	69.4 ± 1.52^{i} j	101.6 ± 2.30^{bc}	106.2 ± 1.30^{ab}	49.6 ± 1.52^{l}

[a-l] in the column indicate significant differences ($p<0.05$). The small letter "a" indicates a higher number compared to group "b", while "ab" indicates a non-significant difference ($p> 0.05$).

On day 10, the number of fibroblasts generally began to decline in the K+, P1, and P2 groups, but the P3 group continued to show the highest number of 106.2±1.30, indicating a more sustained proliferation process. The increase in the number of fibroblasts in the group that received nano chitosan treatment, especially 750 ppm (P3), showed the effectiveness of chitosan in accelerating tissue regeneration and strengthening healing tissue in the proliferation phase. Furthermore, on day 14, almost all groups experienced a decrease in the number of fibroblasts, with the lowest value in the P3 group at 49.6±1.52, indicating the onset of the remodelling phase and the completion of the healing process.

Microscopic observation of HE staining results increased from day 1 to day 3 in all groups, with groups P3 and P2 showing the highest number of fibroblasts on day 3. Meanwhile, a decrease in fibroblasts began to appear on days 10 and 14 as a sign of entering the remodelling phase. Figure 2 shows microscopic observation of tissue with HE staining, which can be seen in Figure 2.

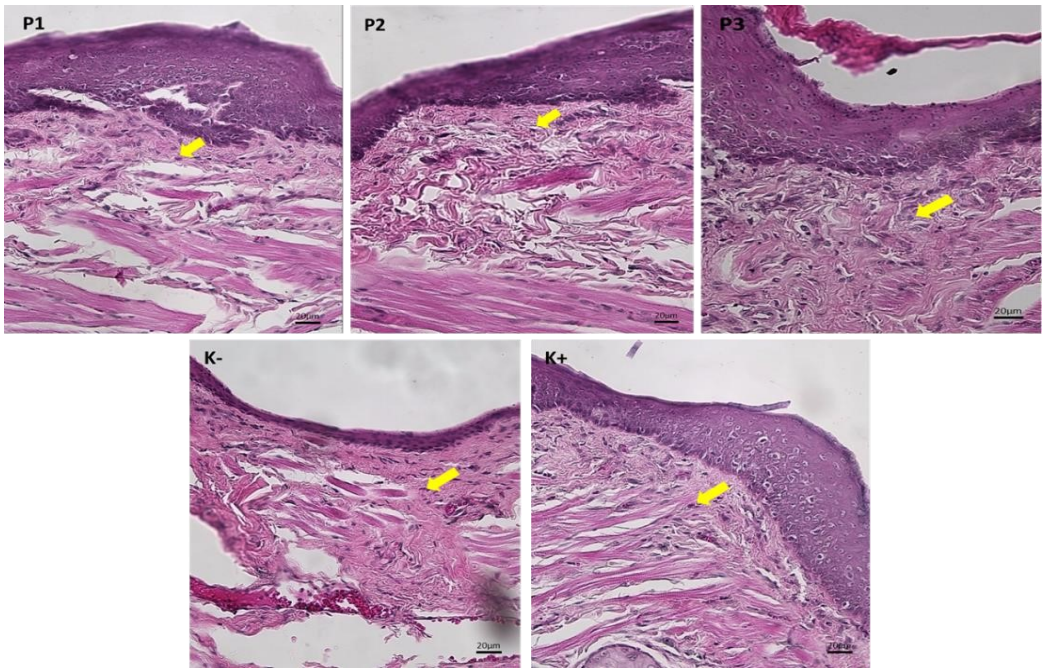
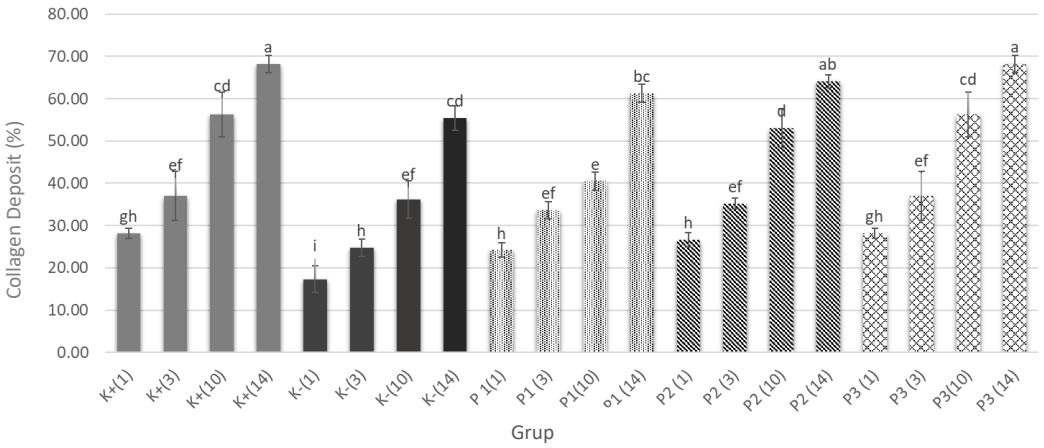


Figure 2. Microscopic observation of fibroblasts with HE staining on day 14; Nano chitosan 3000 ppm (P1), Nano chitosan 1500 ppm (P2); Nano chitosan 750 ppm (P3); Negative control (-); Positive control (+). The yellow arrow indicates the observation of fibroblasts at 200X magnification.

Observation of Collagen Deposition

The collagen deposit observations showed significant results in all groups over the observation period, on days 1, 3, 10, and 14. On the first Day, the percentage of collagen deposits in all groups was still relatively low, with the highest values in groups P3 and P2 at 28.22% and 26.69%. Group K had the lowest collagen deposit value at 17.31%.

On day 3, all groups experienced an increase in collagen deposition. The highest increase occurred in the nano chitosan treatment groups, particularly P3 and P2, which reached more than 35%, indicating that the tissue response to nano chitosan therapy was rapid. On day 10, group P3 showed the most significant increase, reaching more than 56%, followed by P2 (53.09%), which was consistently higher than the control group. Group K, as a negative control, had the slowest rate of increase and remained the lowest on all observation days. On day 14, the differences between groups became more apparent. Group P3 showed the highest percentage of collagen deposition, reaching almost 75%, followed by P2 and P1, which also showed high numbers, while K+ and K- were below them (Figure 3).



[a-i] in the column indicates a significant difference ($p < 0.05$). The lowercase letter "a" indicates a higher number compared to group "b", while "ab" indicates non-significant difference ($p > 0.05$).

Figure 3. Results of collagen deposit percentages in the study groups.

The results of collagen fibre deposition observations increased gradually from day 1 to day 14 in all groups. Group P3 showed the highest percentage of collagen fibre deposition, at 74.54% on day 14. Meanwhile, the negative control group showed the lowest collagen fibre deposition at each observation time. The results of microscopic observation of collagen deposition can be seen in Figure 4.

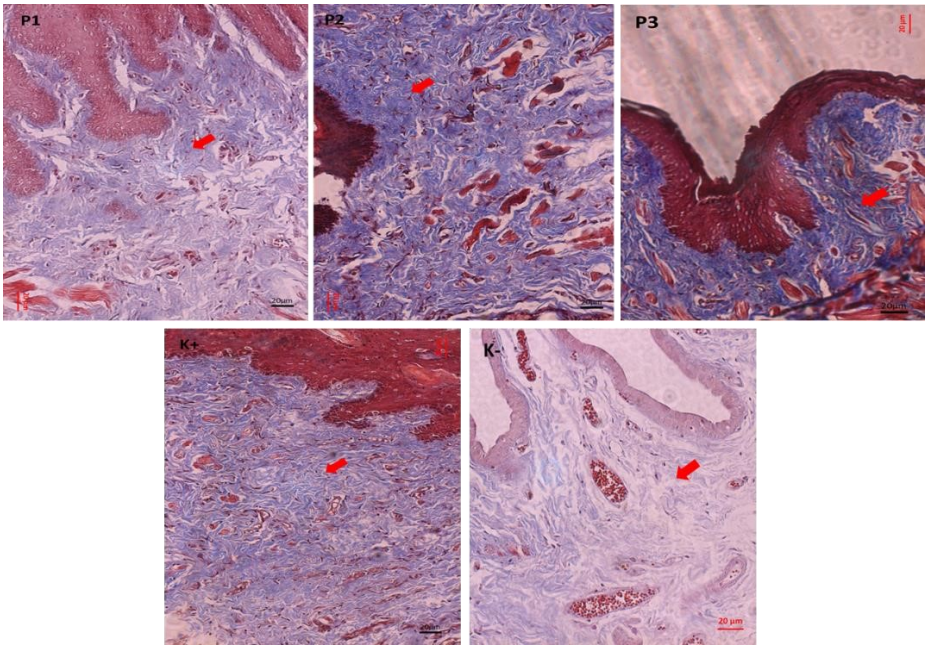


Figure 4. Microscopic observation of collagen deposits with Masson's Trichrome staining on day 14; Nano chitosan 3000 ppm (P1), Nano chitosan 1500 ppm (P2); Nano chitosan 750 ppm (P3); Negative control (-); Positive control (+). The yellow arrow indicates the observation of fibroblasts at 200X magnification.

Discussion

Research shows that topical application of *Xylotrupes gideon* nano chitosan on cheek mucosa abrasions in rats significantly accelerates wound closure. Measurements of wound length in the group given nano chitosan, especially at a concentration of 750 µg/mL, showed that the wounds formed shrank or decreased in size the fastest compared to the negative and positive control groups. At the end of the observation period, all groups showed completely closed wounds, but the healing rate was more apparent in the nano chitosan group. Chitosan has active compounds that modulate wound healing (Feng et al.,

2021). Chitosan is known to have haemostatic activity that helps stop bleeding quickly by inducing platelet aggregation and accelerating fibrin clot formation in the wound area. Controlled bleeding of wounds will affect the inflammatory process and tissue proliferation, which can proceed more effectively (Feng et al., 2021). In addition, chitosan also acts as an effective antimicrobial agent against bacterial infections that can generally slow down wound healing, keeping the wound clean and reducing the risk of contamination. Chitosan can act as a physical barrier, and its antimicrobial activity can create an optimal environment for new tissue regeneration (Rajinikanth B et al., 2024).

Chitosan in nanoparticle form has bioadhesive and mucoadhesive properties that allow the active substance to remain on the wound surface for a long time, thereby continuously accelerating the healing process with optimal concentration (Feng et al., 2021). In addition, the small size of chitosan nanoparticles allows them to easily penetrate the mucosal layer and maximize interaction with target cells and tissues beneath the wound surface (Rajinikanth B et al., 2024).

The number of fibroblasts increased significantly after the administration of nano chitosan, peaking on day 3 in the 750 ppm and 1500 ppm concentration groups, indicating more effective fibroblast proliferation stimulation than the control. Fibroblasts are the primary cells responsible for forming the extracellular matrix and producing collagen and other important components in tissue repair (Dong et al., 2025). The increase in the number of fibroblasts in the group given nano chitosan may be due to the ability of nano chitosan to stimulate fibroblast proliferation and migration to the wound area. Chitosan has a chemical structure rich in amino (-NH2) and hydroxyl (-OH) groups, which can interact with fibroblast receptors or growth factors such as *basic fibroblast growth factor* (bFGF), known as the primary stimulant of fibroblast proliferation (Guan et al., 2020). This interaction prevents the degradation of bFGF, thereby increasing its effectiveness in stimulating fibroblast growth (Dong et al., 2019). In addition, nano chitosan also increases the activity of macrophages and neutrophils that produce cytokines and growth factors such as *transforming growth* factor-β (TGF-β) and *platelet-derived growth factor* (PDGF), which indirectly increase fibroblast proliferation and differentiation (Dong et al., 2019; Guan et al., 2020), thus triggering a cellular response that accelerates the proliferation phase of wound healing (Prastika et al., 2020). The decrease in the number of fibroblasts on day 14, especially in the group receiving nano chitosan, indicates the onset of the remodelling phase. In this phase, fibroblasts decrease because most have already played a role in forming connective tissue, and collagen matrix reorganization begins. The decrease in the number of fibroblasts also indicates that the wound has reached the advanced healing stage with the formation of stable tissue (Cialdai et al., 2022).

Collagen deposition, an important parameter, indicates the quality of wound repair because collagen is the main protein that provides strength and integrity to new tissue (Benito-Martínez et al., 2025). Collagen deposition showed a gradual and significant increase in the group receiving nano chitosan, especially at a concentration of 750 ppm on day 14. Chitosan is known to induce collagen synthesis through an indirect mechanism as a bioactive biomaterial. The positive charge of chitosan interacts with the fibroblast membrane, thereby strengthening adhesion and proliferation (Kim et al., 2023). This interaction activates the integrin–FAK/Akt and MAPK/ERK signalling pathways, which promote the expression of type I and III collagen genes (Pang et al., 2023). In addition, the antioxidant activity of nano chitosan also helps reduce oxidative stress in the wound area, which plays an important role in protecting fibroblasts and collagen from free radical damage, thereby accelerating tissue repair (Loo et al., 2022).

In the wound healing process, nano chitosan can accelerate the healing of incision wounds through several mechanisms. First, nano chitosan stimulates proliferation by enhancing cell-ligand interactions, maintaining the stability and activity of bFGF towards fibroblast migration and proliferation (Loo et al., 2022). Second, it stimulates the release of TGF-β, PDGF, and VEGF, which play a role in angiogenesis, cell migration, and matrix synthesis (Dev et al., 2025). Thirdly, it has antimicrobial and antioxidant activities that can inhibit infection and reduce ROS (which can damage tissue if excessive) (Ukaegbu et al., 2025). Mucosal tissue, increasing local penetration and therapeutic effectiveness (Ukaegbu et al., 2025). Finally, the gel form of chitosan can maintain wound moisture, ensuring that cell-healing activity is not hindered (Rajinikanth B et al., 2024). Fourthly, the size of the nanoparticles makes it easier for chitosan to penetrate.

CONCLUSIONS

Topical application of nano chitosan *X. gideon* to cheek mucosal abrasions in rats significantly accelerated the wound healing process, as indicated by faster wound length reduction, increased number of fibroblasts in the proliferation phase, and increased collagen fiber deposition, signifying optimal tissue regeneration. A concentration of 750 ppm yielded the best results compared to higher concentrations.

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