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THE USE OF GREEN CLAM SHELL (*Perna viridis*) AND GOLDEN SEA CUCUMBER (*Stichopus hermanii*) GEL IN HEALING APICAL FENESTRATION WOUNDS THROUGH TGF-β EXPRESSION ANALYSIS

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ABSTRACT

Background: Apical fenestration denotes a cortical plate opening in the alveolar bone resembling a window shape and affects the apical tooth root area. The shell of green clams (*Perna viridis*) and golden sea cucumbers (*Stichopus hermanii*) possess antibacterial properties, attributed to high levels of glycosaminoglycans and calcium, aid wound healing by reducing inflammation and promoting bone regeneration.

Aim: This study seeks to validate the efficacy of a gel containing a blend of green clam shell hydroxyapatite and golden sea cucumber in healing apical fenestration wounds, measured through increased TGF-β1 expression.

Methods: A laboratory experimental research and clinical trial were conducted, utilizing a posttest only control group design involving 27 Wistar samples. Rodents were distributed into three categories: negative control, positive control (*aloe vera* gel application), and treatment (gel with green clam shells and golden sea cucumbers). Sacrifice occurred over 3, 7, and 14 days, with immunohistochemical examination for data analysis.

Results: One-way ANOVA and post hoc LSD ANOVA tests revealed a significant increase in TGF-β1 expression ($p<0.05$) in both gingival soft tissue lesions and alveolar hard tissue defects within the treatment group. The gel effectively facilitated tissue re-epithelialization and bone remodeling in healing apical fenestration wounds.

Conclusion: The application of a gel containing a combination of green clam shell and golden sea cucumber proved effective in healing apical fenestration wounds in *Rattus norvegicus* (Wistar rat) teeth, as evidenced by the increase in TGF-β1 expression.

Keywords: *Apical fenestration, green clam shell, golden sea cucumber, TGF-β*

INTRODUCTION

Fenestration, a defect in the alveolar bone's cortical plate resembling a window, can arise from various physiological or pathological processes. When occurring at the root's apical region, it's termed apical fenestration. Concurrent mucosal fenestration and apical fenestration could result in the exposure the root tip to the oral environment. Studies indicate that gingival wounds and hyperplastic tissue surrounding the area typically undergo spontaneous healing post-tooth extraction.[1,2,3]

Impaired wound healing in the oral cavity can stem from trauma, prolonged inflammation, and post-operative issues. This intricate process involves interactions among various cells and chemical mediators, inclusive of the transforming growth factor-β (TGF-β) which is consequential in this mechanism. TGF-β regulates the inflammatory phase. Additionally, it modulates angiogenesis in terms of cell movement, influx and the maturing of endothel.[4,5,6]

The green clam shell (*Perna viridis*) is renowned for its high quality, comprising approximately 95% calcium carbonate ($CaCO_3$) alongside organic substances and oxides like SiO_2, MgO, and SO_3. Through calcination, the calcium carbonate in green clam shells can serve as an antibacterial agent.[7,8] Similarly, the golden sea cucumber (*Stichopus hermanii*) is widely utilized in traditional medicine across various regions, renowned for its efficacy in wound healing. Rich in minerals like calcium, golden sea cucumbers aid in reducing inflammation and expediting the bone remodeling process. [9,10]

In according with the explanation above, the author intends to conduct research to look at the combination of green clam shell extract and golden sea cucumber gel in healing apical fenestration wounds of primary teeth through the analysis of TGF-β1 expression.

MATERIALS AND METHODS

This research is a laboratory experimental research and clinical trial with a post-test only control group design with 27 wistar samples. The rodents are distributed into categories: negative control group, positive control group (*aloe vera* gel application), treatment group (combined green clam shells and golden sea cucumbers gel application) which will be sacrificed within a period of 3, 7 and 14

days. Immunohistochemical examination is performed on experimental animals to analyze data. This research has obtained ethical approval from the Ethic Section of Dentistry Faculty, Hasanuddin University under Ethical Approval Letter No. 0124/PL.9/KEPK FKG-RSGM UNHAS/2023.

Process for Making Hydroxyapatite from Green Clam Shell Powder

Green clam shell waste is cleaned then dried in the sun. The shell is size-reduced through ball milling, filtered with a 100 mesh sieve, and undergo calcination at 1000°C for 5 hours using a Muffle Furnace type 6000. The green clam shell extract is characterized, prior and subsequential of the calcination.

The resulting calcined green clam shell extract is combined with 100ml purified water at a temperature of 40°C, added drop by drop to 100ml of 0.3 M $(NH_4)_2HPO_4$ solution, followed by stirring at 60°C for 30 minutes using magnetic stirrer. Precipitation weighing was carried out using an analytical scale after filtration with Whatman 42 filter paper and oven drying at a temperature of 110°C for a duration of 5 hours. Lastly, the precipitate is burned at at 700°C for 1 hour using a Muffle Furnace type 6000 to obtain hydroxyapatite $Ca^{10}(PO_4)6(OH)_2$.

Process for Making Golden Sea Cucumber Extract

Golden sea cucumbers are cleaned and sun-dried for 7 days, then in the oven at 40-50°C for 3-5 days, then cut into small pieces. Homogenization and extraction by maceration with 1:3 volume ratio of methanol is executed on about 700g of golden sea cucumbers. Prior to stirring by orbital shaker, leaving of the mixture for 72 hours is done with solvent replacement every 24 hours. The resulting extract (macerate) undergoes filtration and concentration using a rotary evaporator at 40°C until an extract is obtained. Supernatant for each sample is generated, followed by centrifugation for 10 minutes then storing at 10°C.

Process for Making Gel

A concentration of green clam shell and golden sea cucumber shells of 0.8% each is utilized in 100 ml of distilled water with 0.2 g of Na-CMC as the gelling agent. The extraction of green clam shells and golden sea cucumbers is done separately, by expanding the Na-CMC with warm water gradually then mixing it with propylene glycol and glycerin. Finally, add the extract and stir the remaining water until homogeneous.

Green clam shell (*Perna viridis*)

Golden sea cucumber (*Stichopus hemanii*)

Figure 1. Process of making shell gel from green clam and golden sea cucumbers.

Treatment of Experimental Animals

a) Creation of defects in the gingiva and alveolar bone of *Rattus norvegicus*

- *Rattus norvegicus* was anesthetized using 10% Ketamine HCL solution. After 10-15 minutes, limping and slowed down movements were seen in the rats, and observation of anterior teeth's apical approximate location was conducted.
- Defects were created in the gingiva and alveolar bone around the apical area of the anterior teeth using low-speed handpiece with small round bur. The mucosa and alveolar bone are drilled through the periosteum until the apical of the tooth is visible. Irrigate with 0,9% NaCl. Observe the clinical condition of the oral cavity and changes in the movements of *Rattus norvegicus*.

b) Gel Application

- The total sample comprised 27 mice, divided into three groups, namely 9 treatment groups, 9 positive control groups and 9 control groups.
- Place the green clam shell gel and golden sea cucumber gel on a glass plate in a 1:1 ratio, stir until homogeneous using a spatula.
- The treatment group then applied 1 ml of a concoction of green clam shell and golden sea cucumber gel to the defect area in the gingiva and

alveolar bone slowly using a small excavator, the positive control group applied 1 ml of *aloe vera* gel to the defect area in the same manner and the control group received no application.

- *Rattus norvegicus* was sacrified on days 3, 7, and 14 post-treatment, with 3 animals from each group. Euthanasia was performed using ether put in a jar. Rats were fixed on a work table and decapitation was carried out, separating the cranium from the mandible. Subsequently, the tissue underwent cutting and rinsing in distilled water. Each specimen was then submerged in a container filled with 10% formalin buffer and appropriately labeled.
- The tissue taken is then prepared for histological examination.
- The preparations were then stained using Harris Hematoxylin Eosin (HE) staining.
- The preparations were then observed under a binocular light microscope with a magnification of 1000 times to see the amount of TGF-β1.

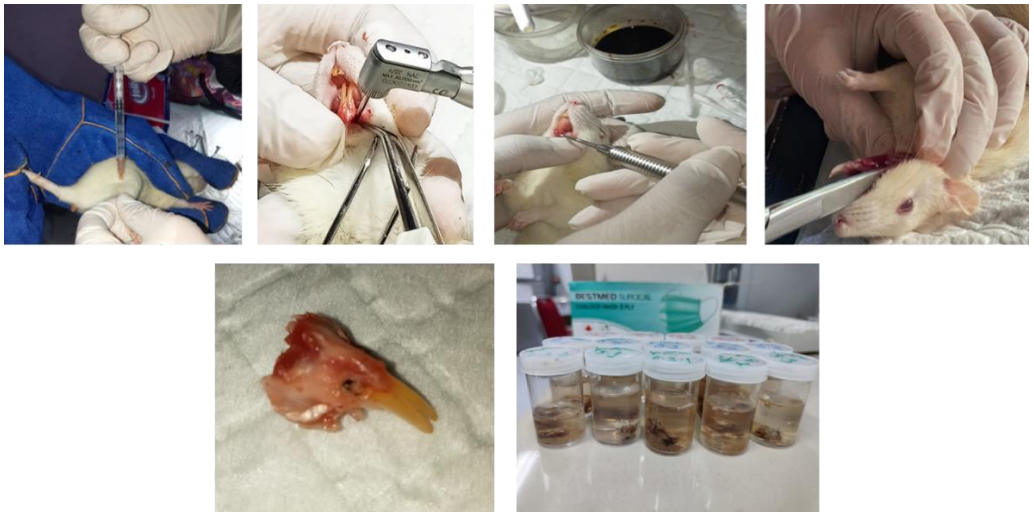


Figure 2. Process of treating experimental animals.

c) Statistical Analysis

The normality of the data was verified through the Shapiro-Wilk test ($p > 0.05$), indicating homogeneous and normally distributed data. Parametric tests, including one-way ANOVA and post hoc LSD ANOVA, were then conducted to assess TGF-β1 levels in soft tissue (gingival) and hard tissue (alveolar) lesions

across the three groups.

RESULT AND DISCUSSION

The healing process of gingival and alveolar wounds in this study can be seen from the HE examination and observations regarding the expression of TGF-β1 can be seen from the Immunohistochemistry (IHC) examination. Immunohistochemical techniques, employing antibodies against TGF-β1 from Santarcruz Biotech, were utilized to observe the distribution of TGF-β1. By using immunohistochemical results calculation techniques as in Soini, Y., Paakko, P. and Lehto, V-P. (1997); Pizem, J. and Cor, A., (2003) modified for bone tissue, this study has completed counting the number of osteoblast cells in soft tissue and hard tissue, which are positive for NFkB (TGF-β1+ cells) which marked by brown color existence in the cell cytoplasm, an arrow in the image (1000x magnification). A raise in TGF β positive osteoblast cells (TGF-β1+) in the K+ and P groups compared to the K- group were observed. And it appears that in group P the number of TGF-β1 positive osteoblast cells (TGF-β1+) was higher compared to group K+.

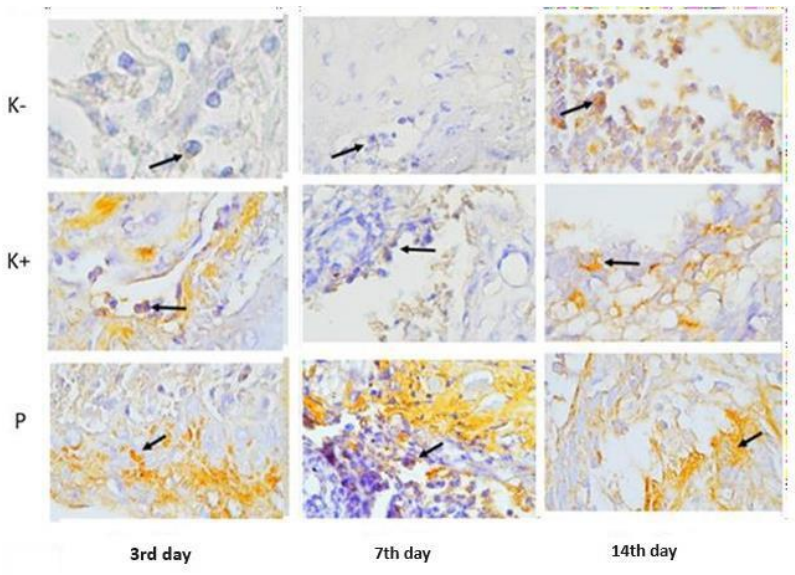


Figure 3. Microscopic photos of TGF-β1 expression of inflamed *Rattus norvegicus* gingiva on days 3, 7 and 14 at 1000x magnification.

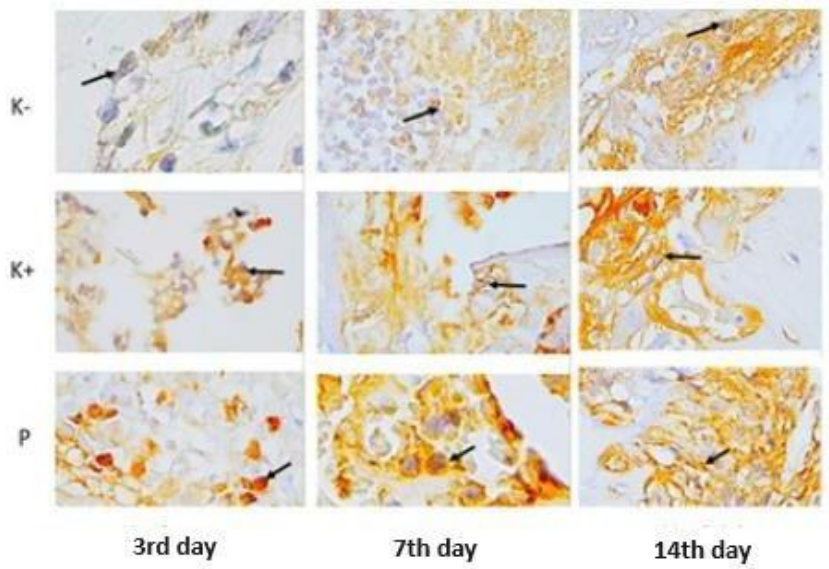


Figure 4. Microscopic photos of inflamed alveolar TGF-β1 expression of *Rattus norvegicus* on days 3, 7 and 14 at 1000x magnification.

Research of TGF-β1 expressiveness after the application of treatment group materials, positive and negative control groups in the area of the lesion and defect in the gingiva and alveolar bone of the *Rattus norvegicus* was conducted in July-August 2023.

Table 1. Comparison of the amount of TGF-β1 expression for each treatment group based on observation time.

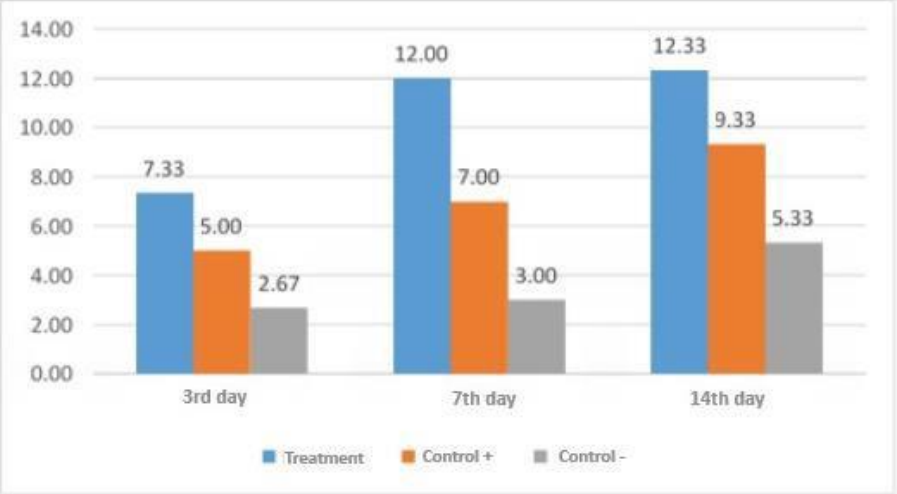
	Treatment Group	3rd day (mean ± SD)	7th day (mean ± SD)	14th day (mean ± SD)	*p-value*
Gingival	Treatment	7.33 ± 1.527	12.00 ± 1.000	12.33 ± 1.527	0.007*
	Control +	5.00 ± 1.000	7.00 ± 1.000	9.33 ± 1.527	0.013*
	Control -	2.67 ± 0.577	3.00 ± 1.000	5.33 ± 1.527	0.049*
Alveolar	Treatment	8.33 ± 1.527	11.67 ± 1.527	13.67 ± 1.527	0.014*
	Control +	4.00 ± 1.000	5.00 ± 1.000	9.33 ± 1.527	0.004*
	Control -	3.00 ± 1.000	4.33 ± 1.527	6.67 ± 1.527	0.044*

*significant one-way ANOVA test ($p<0.05$)

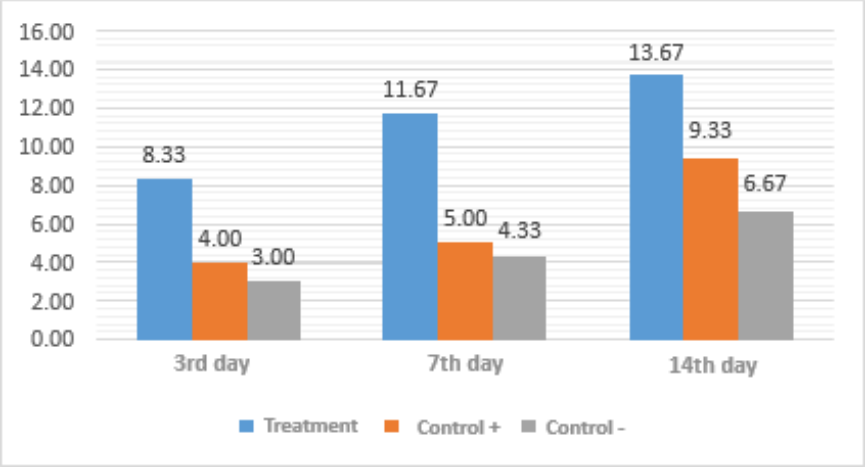
In Table 1, the average gingival TGF-β1 levels in the treated group increased on days 3, 7, and 14. Statistical analysis utilizing one-way ANOVA indicates a notable difference ($p<0.05$) in the quantity of gingival TGF-β1

expression among the groups. Specifically, on day 3, 7, and 14, the treatment group exhibited a p-value of 0.007, the positive control group.

The average alveolar TGF-β1 levels in the treated group rose on days 3, 7, and 14. Statistical analysis revealed a significant disparity ($p<0.05$) in alveolar TGF-β1 expression among the groups. Specifically, the treatment group exhibited a p-value of 0.014, p-value of 0.004 was observed for the positive control group, and the negative control group displayed a p-value of 0.044.had a p-value of 0.013, and the negative control group showed a p-value of 0.049.



Graph 1. Mean Gingival TGF-β1 expression in the treatment group based on observation time.



Graph 2. Mean Alveolar TGF-β1 expression in the treatment group based on observation time.

Graph 1 shows the average expression of TGF-β1 in soft tissue lesions (gingiva) while graph 2 shows the average expression of TGF-β1 in hard tissue defects (alveolar) as seen in graph 1 and graph 2 between treatment groups based on time. Observations showed that the highest TGF-β1 expression results on the 3rd, 7th and 14th observation days were the treatment group (green clam shell and golden sea cucumber combination gel), then the second highest were the positive control group (*Aloe vera* gel) and the negative control with the lowest results.

On the results between treatment groups in table 1, application of green clam shells and golden sea cucumbers gel showed a significant increase in TGF-β1 expression according on the observations day 3, day 7 and day 14 as a parameter for gingival and alveolar wound healing with a p value <0.05. The high protein (glycosaminoglycan, heparan sulfate, hyaluronan, proteoglycan, saponin, omega-3, calcium, zinc) and collagen content of golden sea cucumbers provides an osteoinduction effect in bone regeneration. This aligns with findings from Sari RP, et al (2019)[11], indicating that golden sea cucumbers can accelerate bone formation process 14 days after rat tooth extraction.

Sari RP et al. (2017)[12] investigated the expression of bFGF and new blood vessels created during bone defect healing using golden sea cucumbers combined with blood cockle shells. In a separate study by Sari RP et al. (2021)[13], a scaffold composed of golden sea cucumber and blood cockle shells demonstrated effectiveness in increasing CD44 and IL-10 expression, leading to reduced osteoclast activity in the healing process of an extracted tooth's socket. Additionally, Damaiyanti DW et al. (2019) found that the most significant reduction in ulcer diameter occurred in group 1 treated with golden sea cucumber gel at an 80% concentration.

The active compound content in this treatment group was higher than the other treatment groups, causing the wound area to be exposed constantly until the 7th day of treatment.[14] There were no notable differences among the treatment, positive control, and negative control groups on days 3 and 7. However, on day 14, no notable difference was found between the treatment group and positive control group. This is thought to be influenced by the length of exposure to the material, thus affecting its effectiveness. During the process of wound healing,

the body needs an extracellular matrix.

The extracellular matrix plays a role in organizing and creating the framework for many wound healing processes. Based on research by Rizal (2012), the glycoprotein content in golden sea cucumbers is 3,18%. Adhesive glycoproteins, like fibronectin in the interstitial extracellular matrix and laminin in the basal lamina, play a key role in binding matrix elements and attaching the matrix to cell surfaces through integrins. Collagen constitutes the most abundant fibrous element in the extracellular matrix.[15]

Golden sea cucumber water extract has been proven to be able to accelerate the healing of traumatic ulcers by increasing type I collagen production and reducing the ulcer diameter. The results of this study are supported by preliminary research with similar subjects.[16] In addition, research by Masre SF, et al., (2010) which used sulfated GAGs (glycosaminoglycans) from golden sea cucumber extract showed that there was an increase in contraction through increased collagen synthesis which would improve wound closure. by reducing the diameter of the ulcer.

In this study, observations on the test animals were carried out at time intervals on the 1st, 6th and 12th days after treatment, different from research that has been carried out, namely observations on the 3rd, 7th and 14th days. [17] Sea cucumbers contain essential minerals, notably iron (Fe), copper (Cu), calcium (Ca) and magnesia (Mg). Fe is required for the hydroxylation of proline and lysine. Both Fe and Zn are essential for collagen synthesis, tissue growth and bringing oxygen to wounds. Cu plays a role as a cofactor in cytochrome oxidation, for the cytosolic antioxidant SOD and for collagen cross-linking. Ca plays a role in regulating blood clotting, while Mg can increase the number of fibroblasts so that collagen synthesis also increases.[15]

Sea cucumbers, rich in cell growth factor (CGF), expedite wound healing by stimulating cell regeneration. CGF encompasses various types, including TGF, PDGF, FGF, EGF, HGF, and VEGF, each playing a distinct role in the healing process.[15] Previous research highlights that applying a combination gel of green clam shells and golden sea cucumbers alters TGF-β1 expression in soft tissue lesions and alveolar bone defects, further underscoring their potential in wound healing applications.

The groups treated with green clam and golden sea cucumber, along with the positive and negative control groups, exhibited notable variances in TGF-β1 production levels on days 3, 7, and 14 ($p<0.05$). This shows that green clam shell and golden sea cucumbers have proven to be the most effective in the course of wound healing in *Rattus norvegicus*. This aligns with the findings of the research by Sari RP et al. (2019) who researched the effectiveness of a combination of blood clams (*Anadara granosa*) and golden sea cucumbers and found that the combination could accelerate the formation of woven bone on the fourteenth day after tooth extraction to prevent alveolar bone resorption (socket healing).[11]

High calcium ion content of green clam shells plays a pivotal role in wound healing by regulating inflammatory cell infiltration, fibroblast proliferation, and keratinocyte migration via the transduction route of Mitogen Activated Protein Kinase (MAPK) signal. The main molecule of the MAPK pathway promotes angiogenesis and enhances neutrophil adherence to endothelial cells, bolstering their antibacterial efficacy.

Based on the research results of Mediarman et al., the CaO value contained in green clam shell extract is quite high at 99,62%.[18,19] In addition, in another study by Usman MR et al., (2020), the optimal CaO crystals were determined based on their $\chi 2$ value, with the CaO crystals sourced from green clam shells with a particle size of 96.6566 nm.[20] Application of *Aloe vera* gel was also carried out on test animals to compare the results of the wound healing process between treatment groups.

Aloe vera, renowned for its medicinal properties, is utilized in treating and preventing oral diseases due to its anti-inflammatory, antimicrobial, and cell-regenerating attributes. Its efficacy extends to tissue engineering, as alveolar cells promote migration, proliferation, and growth. It also boasts biodegradability, biocompatibility, and low toxicity. [21,22]

Application of *Aloe vera* gel accelerates wound closure and increases tensile strength by enhancing cell proliferation. The healing process involves physiological responses like bleeding, blood vessel contraction, inflammation. Improved wound healing is indicated by alterations in epithelial thickness, granulation tissue development, angiogenesis, fibroblast density, collagen fiber formation, and wound contraction.[21,23]

Wound healing relies on the intricate interplay between growth factors and cells at the wound site. TGF-β comprises a family of multifunctional growth factors crucial in this process. TGF-β1, TGF-β2, and TGF-β3 as its isoforms share common functional characteristics and are involved in various phases of wound healing, from inflammation to the extracellular matrix reconstruction. Furthermore, TGF-β plays a crucial role in controlling wound re-epithelialization and facilitating connective tissue regeneration, demonstrating its essential role in the wound healing process and scar formation.[24]

CONCLUSION

Based on the conducted research, it is evident that the combination gel of green clam shell and golden sea cucumbers significantly increases TGF-β1 expression in both soft tissue lesions and hard tissue defects. This gel demonstrates efficacy in healing apical fenestration wounds through tissue re-epithelialization and bone remodeling processes, indicating its potential as an alternative to *Aloe vera* gel or conventional medications.

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