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# Stem cells from human exfoliated deciduous teeth secretome hydrogel combined with α-mangostin for periodontal bone regeneration: In vivo study

Mohammed Ahmed Aljunaid [a,b], Rini Devijanti Ridwan [c,*], Hendrik Setia Budi [c], Yuliati [c], Huda Rashad Qaid [b], Rubbig Diferly Mahendra [d], Chanaya Miranda Riveira [d], Abumabdian Nasar Aqrabi [b], Ananda Firman Putranto [e], Farouk Al-Ghazaly [b], Osamah Mohammed Al-Kawri [b]

[a] Department of Oral and Dental Medicine, Faculty of Medicine, Taiz University, Yemen
[b] Faculty of Oral and Dental Medicine, Al-Saeed University, Yemen
[c] Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Indonesia
[d] Faculty of Dental Medicine, Universitas Airlangga, Indonesia
[e] Department of Orthodontia, Faculty of Dental Medicine, Universitas Airlangga, Indonesia

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ABSTRACT

*Objectives:* Periodontitis is a long-term inflammatory condition that results in the gradual loss of bone tissue surrounding the teeth. Cells derived from the pulp of human exfoliated deciduous teeth, also known as SHED cells, are very valuable in regenerative medicine due to their ease of access, rapid multiplication, and the ability to develop into multiple cell types. Additionally, medicinal plants such as mangosteen, which are rich in α-mangostin, show promise for periodontitis treatment. This compound, possessing potent anti-inflammatory and antioxidant properties. The purpose of this study was to investigate the molecular mechanisms of SHED secretome-loaded hydrogel combined with α-mangostin on the periodontal bone regeneration processes in a rat model.

*Material and method:* Sixty Male Wistar rats (n = 12 per group) were divided into five group: Control positive, Control negative, Treatment I, Treatment II, and Treatment III. After treatment, rats were humanely euthanized at three distinct time points (7, 14, and 21 days). Mandibular sections were then collected and analyzed using immunohistochemistry to quantify the expression levels of TNF-α, IL-10, and BMP-2. One-way ANOVA and a post hoc test ($P < 0.05$) were used to assess the data.

*Result:* The result showed decrease in TNF-α expression, and a significant increase in the expression of IL-10 and BMP-2 at 7th, 14th, and 21 days.

*Conclusion:* The combination of hydrogel SHED secretome α-mangostin effectively modulates the inflammatory environment by reducing TNF-α level and enhancing IL-10 production, thereby promoting the expression of the osteogenic factor BMP-2. This finding suggests an effective strategy for periodontal bone regeneration processes.

## 1. Introduction

Periodontitis, an inflammatory disease caused by bacterial infection of the dental supporting tissue, is a major threat to oral health, affecting up to 20% of the global population. It destroys gingival tissue, bone, and ligaments supporting teeth, leading to tooth loss and impacting quality of life.[1,2] Periodontitis causes the degeneration of the periodontal attachment, which then leads to the loss of bone in the alveolar region and may eventually cause the damaged tooth to fall out.[3,4] Periodontitis treatment typically relies on mechanical plaque removal and antibiotics. However, these conventional methods have limitations: mechanical cleaning may not reach all bacteria, and the use of antibiotics risks

* Corresponding author. Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Jl. Mayjen Prof. Dr. Moestopo 47, Surabaya, 60132, Indonesia.
*E-mail addresses:* mohammed.aljunaid90@gmail.com (M.A. Aljunaid), rini-d-r@fkg.unair.ac.id (R.D. Ridwan), huda.qaid@yahoo.com (H.R. Qaid).

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promoting antibiotic resistance.[1,2]

The use of mesenchymal stem cells (MSCs) in bone tissue engineering is becoming a promising approach for regenerating tissue affected by periodontitis. MSCs (Mesenchymal Stem Cells) have distinctive characteristics that make them optimal for tissue repair, such as their capability to travel to injured areas and to encourage healing, regeneration, and development into other cell types. Consequently, MSCs are extensively being explored for tissue regeneration applications across various fields of medicine. [5–7].

Stem Cells from Human Exfoliated Deciduous Teeth (SHED) are recognized as a particularly promising source of MSCs. SHED offers several advantages over other MSC sources, such as those derived from bone marrow or other dental tissues. Additionally, like dental pulp stem cells (DPSC), SHED have the capacity to differentiate into osteogenic, odontogenic, chondrogenic, adipogenic, and neurogenic cells.[8]

SHED are easily obtained using discarded deciduous teeth, requiring only minimally invasive collection techniques. Furthermore, SHED exhibit superior proliferation and heightened differentiation potential, a benefit attributed to their youthful tissue origin. Collectively, these advantageous properties position SHED as a valuable resource for promoting bone formation and wound healing in regenerative medicine.[6,7]

The SHED secretome, comprising the array of soluble factors secreted by these cells, is emerging as a promising cell-free therapeutic strategy for tissue regeneration. Compared to cell-based therapies, the secretome has a variety of benefits, such as easier collection, simplified storage, and scalable production. For instance, studies have demonstrated its efficacy in promoting cell growth and tissue repair, such as the effective repair of calvarial bone defects in mouse models. This evidence underpins the rationale for cell-free regenerative medicine approaches utilizing the SHED secretome as a viable alternative to conventional cell transplantation.[9] SHED secretomes exhibit therapeutic properties attributed to the presence of various cytokines and growth factors. These properties encompass the promotion of cell migration, proliferation, immunomodulation, and overall tissue regeneration.[10,11]

Despite its stability, the SHED secretome exhibits low retention at the target site. To circumvent this critical limitation, it is imperative to integrate the SHED secretome with appropriate biomaterials. This integration is necessary to achieve the controlled-release kinetics of the bioactive factors essential for effective tissue regeneration.[12] α-mangostin, a major bioactive xanthone derived from the mangosteen fruit, exhibits multiple therapeutic actions relevant to tissue repair. Firstly, it may stimulate the release of fibroblast chemotactic mediators, such as transforming growth factor beta (TGF-β), thereby promoting fibroblast proliferation. Secondly, α-mangostin is a potent anti-inflammatory agent. It suppresses inflammatory processes by inhibiting cyclooxygenase and lipoxygenase enzyme activity and by preventing the gene transcription of nuclear factor kappa beta (NF-κB) in cytoplasmic macrophages, which ultimately reduces pro-inflammatory cytokine synthesis. These anti-inflammatory and antioxidant properties may further contribute to healing by stimulating fibroblast growth factor-2 (FGF-2). Despite these compelling synergistic properties, the application of the SHED secretome in periodontal bone regeneration remains largely uninvestigated, particularly when combined with bioactive compounds like α-mangostin.[13]

Hydrogels are favored in regenerative medicine due to their ease of preparation, low cost, low toxicity, ease of handling, and capacity for the controlled release of therapeutic agents. Specifically, chitosan-based hydrogels are widely applied in cell encapsulation and drug delivery due to their inherent mucoadhesive properties. Combining these polymers, alginate and chitosan (Alg/Chi)-based hydrogels (Alg/Chi-HG) are utilized for various biomedical applications. The Alg/Chi-HG system is known for demonstrating improved mechanical strength, optimized swelling ratios, and favorable drug-release kinetics.[14] Therefore, the Alg/Chi-HG platform represents a promising scaffold for delivering regenerative factors to treat complex tissue defects, such as those caused by periodontitis.[3,4]

Despite the availability of conventional treatments, effective regeneration of periodontal bone remains a major clinical challenge. Thus, innovative biomaterial-based therapies are needed to enhance bone healing. Therefore, this study aimed to investigate the molecular mechanisms of SHED secretome-loaded hydrogel combined with α-mangostin on periodontal bone regeneration processes in a rat model. This was achieved by evaluating the expression of key factors, including Tumor Necrosis Factor-α (TNF-α), Bone Morphogenetic Protein-2 (BMP-2), and Interleukin-10 (IL-10), using immunohistochemistry (IHC).

## 2. Materials and methods

### 2.1. Study design

This study used a post-test-only control group design and a true experimental, laboratory design. A total of sixty male Wistar rats were randomly assigned to five separate groups (n = 12 for each group), as follows: Control positive (No ligature-induced periodontitis with no treatment), Control negative (Ligature-induced periodontitis with placebo treatment), Treatment I (Ligature-induced periodontitis with treatment of Hydrogel + SHED Secretome), Treatment II (Ligature-induced periodontitis with treatment of Hydrogel + α-mangostin), and Treatment III (Ligature-induced periodontitis with treatment of Hydrogel + SHED Secretome + α-mangostin). Each group was further subdivided into three independent subgroups, based on the sacrifice time points at 7, 14, and 21 days, with fours rats assigned to each time point.

### 2.2. Animal model

This study used male Wistar rats weighing 200–260 g and 8–9 weeks of age. The animals were procured from the Animal Resource Unit and housed at the Animal Research Facility, Faculty of Veterinary Medicine, Universitas Airlangga. The rats underwent a 7-day acclimatization period, housed individually in separate cages. They were provided with standard rodent pellets and allowed ad libitum access to drinking water.[15,16]

### 2.3. Preparation of SHED secretome

SHEDs were obtained from the upper premolar teeth of clinically healthy male patients, ranging in age from 7 to 11 years.[17] Premolar teeth were extracted as part of routine orthodontic treatment. Written informed consent was obtained from the parents of all donors. The Universitas Airlangga Research Ethics Commission authorized the study methodology, which included the collection of human dental pulp tissue (Approval No. 297/HRECC.FODM/V/2021). After the extraction was performed in a hygienic manner, the tooth pulp was sent straight to the lab for further analysis. The pulp tissue was treated for cell isolation within 2 h after being kept at 4 ∘ C in 1X phosphate-buffered saline (PBS).[18]

The dental pulp was then separated from the crown and root. Following the separation, the pulp tissue was enzymatically digested for 1 h at 37 ∘ C in a solution containing 3 mg/ml of collagenase type I and 4 mg/ml of dispase. The resultant single-cell suspensions ($1 \times 10\ 4 - 2 \times 10\ 4$ cells/ml) were cultivated using Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), which was supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma Aldrich Inc.). The cultures were incubated and maintained in a humidified environment with 5% CO 2 and 95% air at 37 ∘ C. The primary cultures were kept for a maximum of four passes, with the culture media being replaced every four days to exclude non-adherent cells. Debris was removed from the cell cultures using PBS. Cell detachment and transfer to larger culture dishes were performed using 0.05% trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA). Upon reaching 70%−80% confluence, SHEDs at passage four (P4) were prepared for secretome retrieval.[19]

Upon reaching 70%−80% confluence, the SHED culture medium was aspirated, transferred to a conical tube, and centrifuged. he cell pellet was discarded, and the supernatant was obtained. Following collection, the supernatant was poured into a dialysis tube and tightly sealed on both ends. The supernatant-containing dialysis tube was submerged in a beaker of PBS-based solution and then incubated at 4 ∘ C on a magnetic stirrer plate until the medium color disappeared, which demonstrated sufficient dialysis. The dialyzed solution was subsequently removed from the tube, transferred to a conical tube, and centrifuged again until a pellet was formed. After removing any leftover particles using a 0.22 μm filter, the resultant supernatant was kept in a tube at −20 ∘ C. [20–22].

### 2.4. Preparation of Alg/Chi hydrogel and active agent-loaded hydrogels

The Alginate-Chitosan (Alg/Chi) hydrogel was prepared via a solvent evaporation technique. Initially, 0.5g of sodium alginate (Sigma-Aldrich) was dissolved in 25 mL of distilled water. Separately, 0.1g of chitosan powder (sourced from crab shells; Sigma-Aldrich) was dissolved in 25 mL of diluted acetic acid (prepared by mixing 0.5 mL of glacial acetic acid with 24.5 mL of distilled water). To create a homogenous mixture, the alginate solution (Alg) was gradually added to the chitosan solution and stirred at a steady pace at 45 ∘ C using a magnetic stirrer. Then, 2% glycerol and 5% propylene glycol were added to the resultant mixture. After that, homogenization was performed using an Ultra Turrax homogenizer at 20,000 rpm for 10min. The homogenized solution was centrifuged at 4500 rpm for 5 to 10min to eliminate entrapped air bubbles. This mixture was poured into a Petri dish, levelled, and oven-dried for 5 to 10h until a semi-solid consistency was achieved. The semi-solid hydrogel was then cooled at 4∘C for 1 h.[23] Subsequently, the base Alg/Chi hydrogel was uniformly mixed with the active ingredients: SHED secretomes (16.6 μL) or α-mangostin (250 μg dissolved in 10 μL of 96% ethanol). [24–26] The resulting composite hydrogel was stirred until homogeneous, then dried in the refrigerator for 8 to 10 days at 4∘C.

### 2.5. Induction of experimental periodontitis and treatment protocol

Rats were anesthetized via intramuscular injection using a 2:1 mixed solution of 10% Ketamine and 2% Xylazine, administered at a dose of 0.12 ml/100g of body weight. Following anesthesia, periodontal pathology was induced. Experimental periodontitis was induced by placing a 4−0 silk suture (BBRAUN Silkam®, 7 mm, 45 cm, 3/8 reverse cutting needle, Melsungen, Hessen, Germany). The silk ligature was placed around the mandibular incisors in a figure-eight configuration and secured with a knot on the buccal side. The ligatures were monitored daily throughout the experimental period and replaced immediately if they became loosened or missing. The ligatures remained in place for 14 days. During this period, clinical signs of periodontitis, such as gingival bleeding and swelling, were observed daily, and radiographic analysis was performed. Following the induction phase, the hydrogel treatment was topically administered to all groups around the gingival sulcus of the lower incisors using a syringe. The treatment was applied on the 7th, 14th, and 21st days post-induction. [27–29].

### 2.6. Immunohistochemistry preparation and analysis

The animals were humanely sacrificed at the completion of the experiment. To determine the expression of TNF-α, BMP-2, and IL-10, the anterior mandibles were removed and then prepared for indirect immunohistochemistry labeling. The antigen expression was assessed with monoclonal antibodies against TNF-α, BMP-2, and IL-10. Five randomly chosen fields of view were used to analyze tissue sections through a light microscope (Nikon H600L microscope; Nikon, Japan) at 400 × magnification.[30,31]

### 2.7. Statistical analysis

SPSS version 25 was used to analyze all of the data. The data were presented as Mean ± SD. The Shapiro-Wilk normality test was used, and homogeneity of variances was evaluated using Levene's test. Data were analyzed using one-way ANOVA and post hoc tests (HSD Tukey) at each observation time point (7, 14, and 21 days) to compare differences among the experimental groups. A statistically significant difference was defined as $P < 0.05$.

## 3. Result

TNF-α expression results are presented in Fig. 1. The findings demonstrate a decrease in TNF-α expression levels over the 7, 14, and 21-day experimental period across all groups. In particular, as compared to the other groups, the Treatment III group had the lowest levels of TNF-α expression Fig. 2. The post hoc Tukey HSD test revealed significant differences between the groups. At all-time intervals (7, 14, and 21 days), there was a significant difference in TNF-α expression ($P < 0.05$) between the treatment III group across the control positive, negative Treatment I, and Treatment II groups ($P < 0.001$).

BMP-2 expression results are shown in Fig. 3. These data indicate an increase in BMP-2 expression across all groups over 7, 14, and 21 days. Consistent with the other findings, the Treatment III group displayed the highest BMP-2 expression levels Fig. 4. Moreover, Post hoc analysis (Tukey HSD) revealed a significant difference in BMP-2 expression ($P < 0.05$) at all time points (7, 14, and 21 days) between the treatment III group across the control positive, negative Treatment I, and Treatment II groups ($P < 0.001$).

Fig. 5 shows the results of IL-10 expression. Over the intervals of 7, 14, and 21 days, IL-10 expression levels increased in the studied groups, much like BMP-2. IL-10 expression levels were consistently higher in the Treatment III group Fig. 6. At all-time points (7, 14, and 21 days), post hoc analysis (Tukey HSD) revealed a significant change in IL-10 expression ($P < 0.05$) between the treatment III group across the control positive, negative Treatment I, and Treatment II groups ($P < 0.001$).

## 4. Discussion

This experimental research investigates the molecular mechanism by which a hydrogel containing SHED secretome combined with α-mangostin influences bone regeneration process in a rat model of periodontitis. It is hypothesized that the combined hydrogel formulation promotes bone regeneration process by modulating the expression of key cytokines and growth factors, specifically TNF-α, BMP-2, and IL-10, as evaluated by immunohistochemistry (IHC).

According to the Global Burden of Disease Study, up to 20% of people worldwide suffer from periodontitis, making it the sixth most common disease worldwide.[32,33] The prevalence of periodontitis remains particularly high in Indonesia, with the Riskesdas (2018) report indicating an affected percentage of 74.1% of cases. If left untreated, periodontitis can lead to the formation of deep periodontal pockets, increased tooth mobility, and eventual tooth loss. These sequelae contribute to occlusal instability, reduced mastication and food intake, compromised aesthetics, and ultimately, a reduced quality of life.[1,2]

In 2003, Miura et al. made a significant discovery when they identified a new population of multipotent, proliferative MSCs known as SHED. These cells, which are categorized as dental mesenchymal cells originating from cranial neural crest cells, were first recovered from the spontaneously exfoliated primary incisors of children aged 7 to 8.[17] SHEDs have better proliferation and clonogenic capacity than other dental stem cells.[34,35] The strong capacity of SHEDs to develop into osteo/odontoblasts is a key biological characteristic that makes them perfect for dental tissue engineering.[36] However, the human SHEDs are known to release substances with potent regenerative capabilities. Specifically, bone repair is directly linked to osteogenesis, the

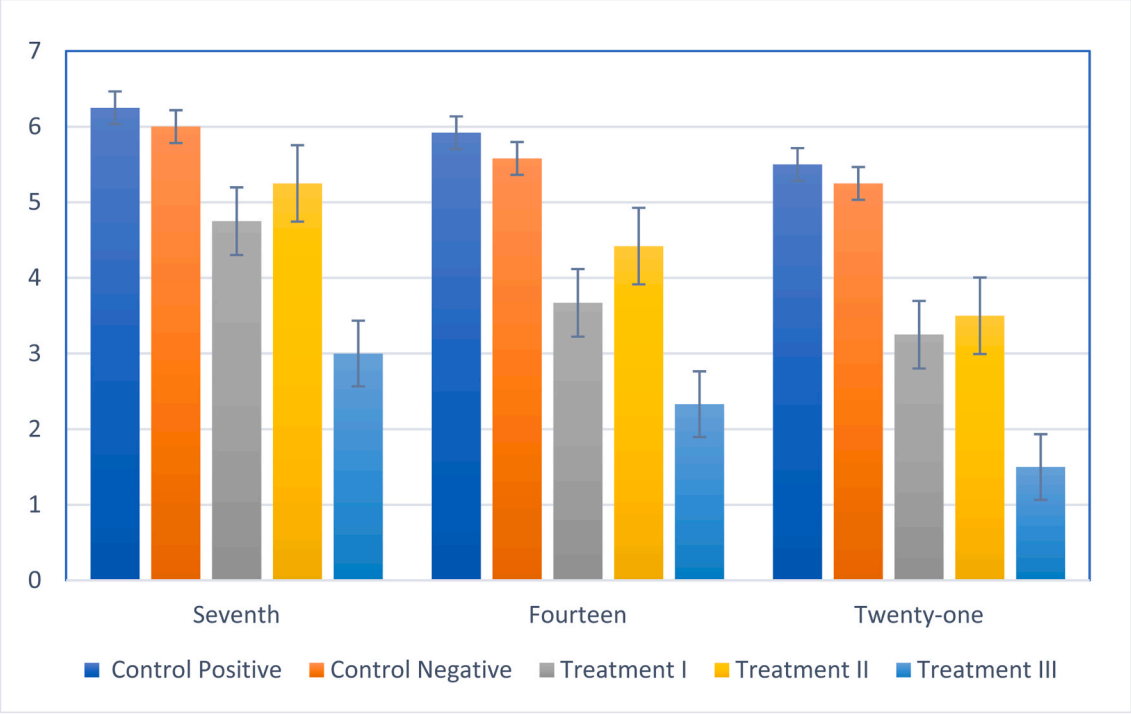


**Fig. 1.** Mean ± SD of the number of TNF- α expression.

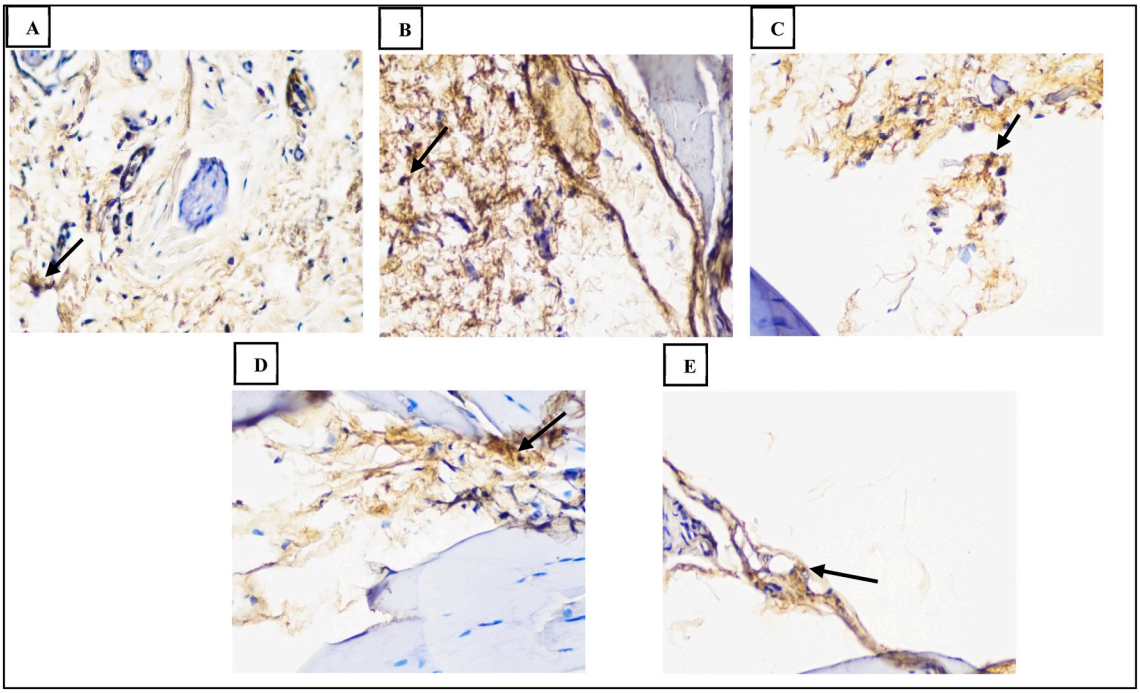


**Fig. 2.** Images of TNF-α expression (Black arrow) (A) Control Positive (B) Control Negative, (C) Treatment I (D) Treatment II (E) Treatment III.

fundamental process of bone formation mediated by osteoblasts.[9,37,38] Moreover, the α-mangostin, a natural xanthone derived from the mangosteen fruit, exhibits potent anti-inflammatory and osteogenic properties, thereby promoting bone cell growth and differentiation.[23,39] Nevertheless, there aren't many direct comparison studies on the therapeutic potential of both SHED secretomes and α-mangostin in promoting bone regeneration process.

TNF-α expression is known to increase significantly during inflammatory conditions such as periodontitis. This upregulation stems primarily from the inflammatory environment created by periodontal pathogens, which stimulate immune cells to release pro-inflammatory cytokines, including interleukin-1 and TNF-α. This study specifically investigated the molecular mechanism effect of hydrogel-based combination of SHED secretomes and α-mangostin on TNF-α expression in a rat model of periodontitis during the bone healing phase. The results of this study provide substantial support for the concept, showing that the combination therapy in Treatment group III group had considerably lower TNF-α expression than all other groups over all observation

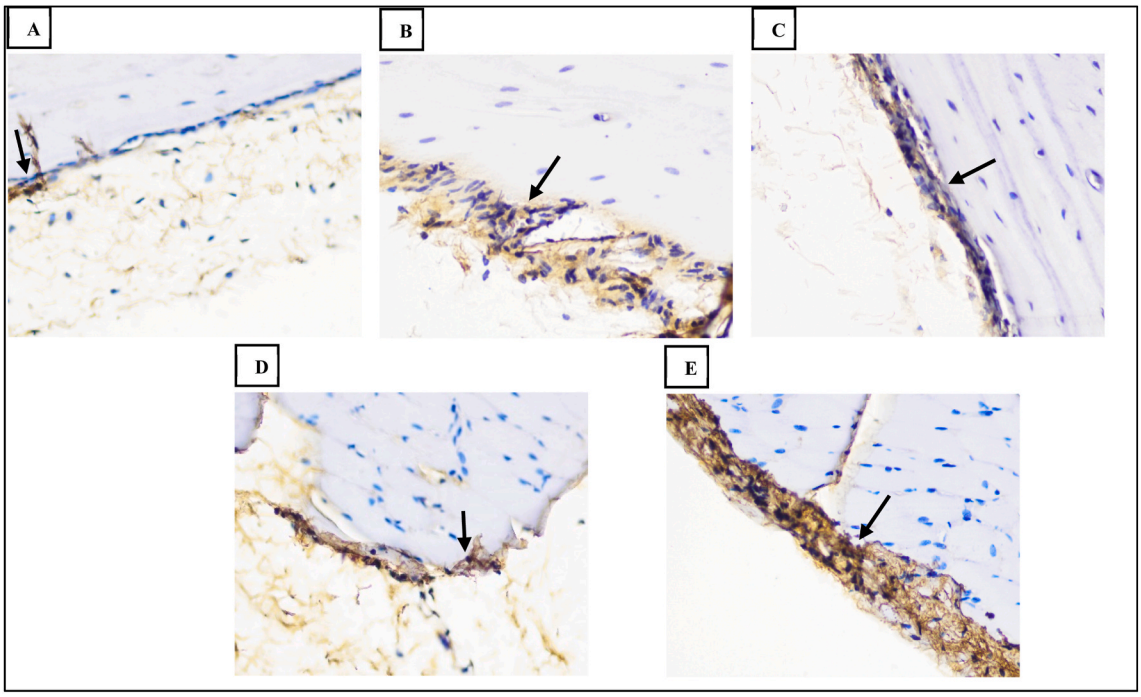


**Fig. 3.** Images of BMP-2 expression (Black arrow) (A) Control Positive (B) Control Negative, (C) Treatment I (D) Treatment II (E) Treatment III.

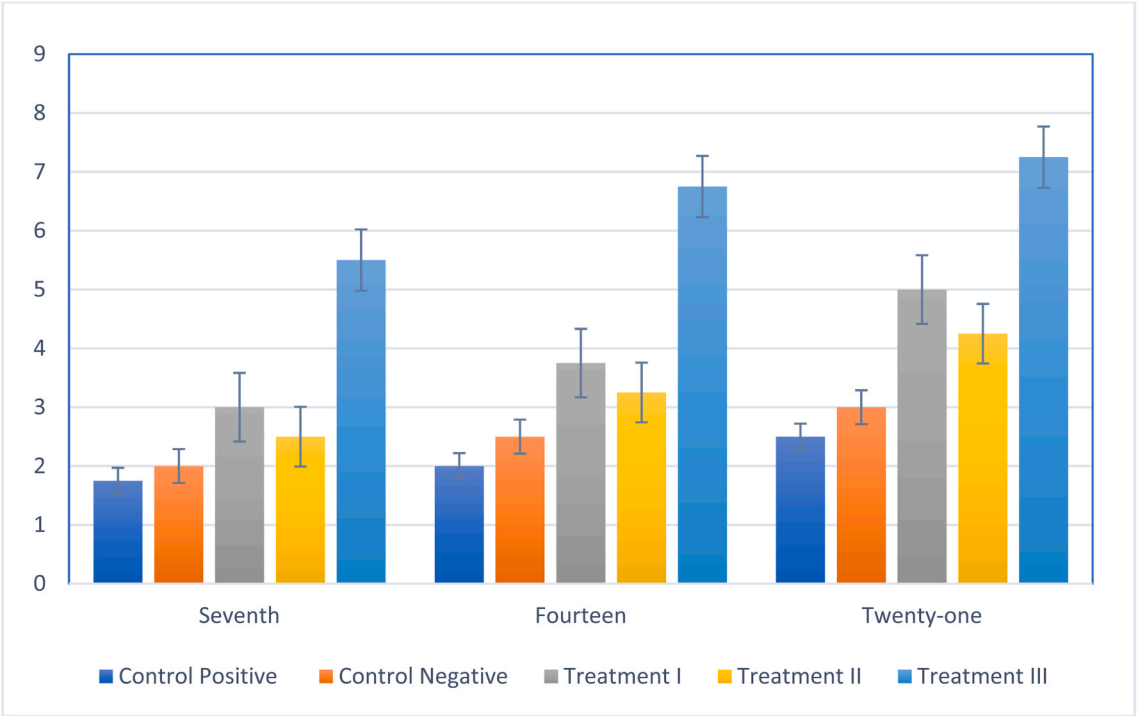


**Fig. 4.** Mean ± SD of the number of BMP-2 expression.

periods (7, 14, and 21 days). The current findings are consistent with previous research that supports the efficacy of hydrogels and dental pulp stem cell-derived secretomes in achieving periodontal regeneration.[9,40,41] The results collectively suggest that the combination of hydrogel SHED secretomes and α-mangostin represents a promising strategy for promoting molecular mechanism of bone regeneration process in periodontal defects.[42]

Moreover, the findings for BMP-2 expression showed that Treatment III significantly increased its expression compared to all other groups. This elevated expression suggests a possible of molecular mechanism effect to enhances the bone regenerative factors, such as BMP-2 promoting factors, present within the SHED secretome.[43] This study results are consistent with previous studies demonstrating that the SHED secretome promotes bone regeneration through the inclusion of growth factors that directly stimulate BMP-2 production. [40,44–47] While α-mangostin has not been directly implicated in BMP-2 upregulation, its

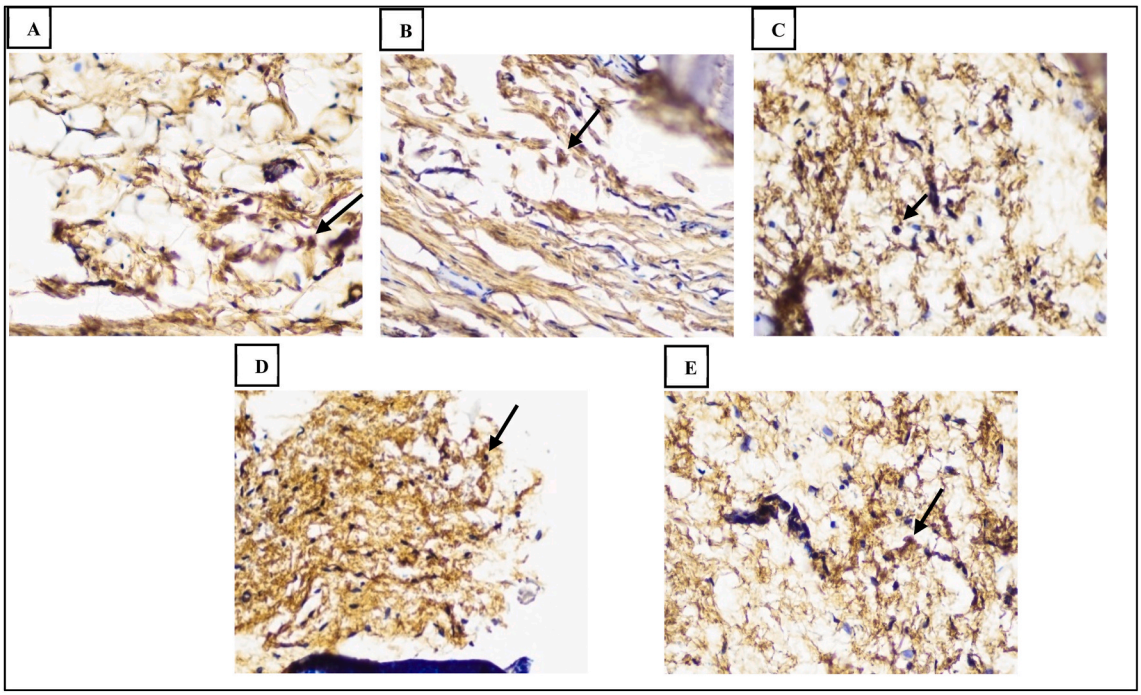


**Fig. 5.** Images of IL-10 expression (Black arrow) (A) Control Positive (B) Control Negative, (C) Treatment I (D) Treatment II (E) Treatment III.

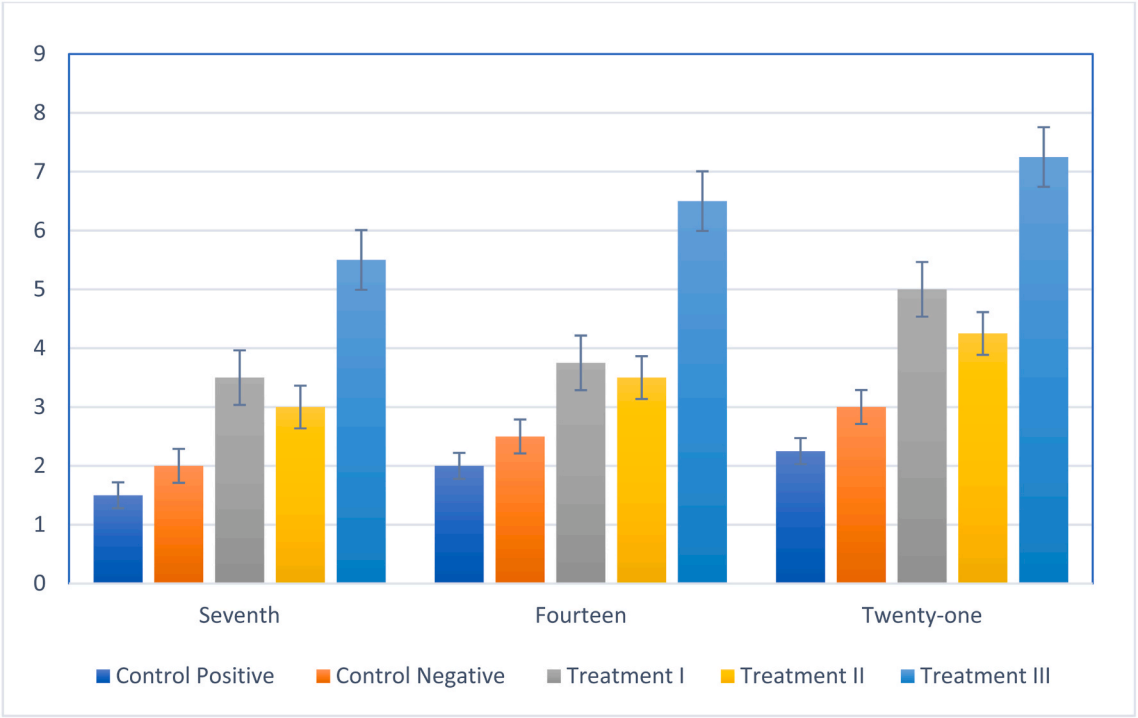


**Fig. 6.** Mean ± SD of the number of IL-10 expression.

contribution may be indirect, stemming from its established anti-inflammatory properties which create a favorable local microenvironment for osteogenesis and bone formation.[45,48]

Inflammation constitutes a complex tissue response to injury that involves a signaling network culminating in endothelial cell activation and subsequent leukocyte recruitment.[49] This leukocyte infiltration is one of the predominant early histological changes and is mediated by a variety of cytokines and adhesion molecules. The inflammatory state of cells is tightly regulated by regulatory cytokines such as Interleukin-10 (IL-10). Produced primarily by activated T cells, IL-10 functions as a key anti-inflammatory cytokine that actively suppresses both immunoproliferative and inflammatory.

The findings of this study are consistent with previous research demonstrating the positive effects of hydrogel SHED secretome combined with α-mangostin on promoting IL-10 expression.[9,39,40] Crucially, this current study extends upon this knowledge by demonstrating that

the combination of SHED secretome and α-mangostin within a hydrogel leads to significantly higher IL-10 expression than either treatment alone. Additionally, previous studies have highlighted the importance of IL-10 in promoting tissue regeneration and suppressing inflammation, which supports the significance of the observed increase in IL-10 expression in this study.[39] This finding suggests that this combined therapeutic approach may be a promising strategy to enhance anti-inflammatory responses and consequently promote the bone regeneration process in periodontitis.

The findings of this study yield several important implications for the development of novel therapeutic strategies for periodontitis. Specifically, the combination of SHED secretome and α-mangostin demonstrates significant potential as a regenerative therapy for promoting osteogenic regeneration in periodontitis. This combined approach may serve as a promising alternative to conventional treatments, with the potential to enhance patient outcomes and quality of life.

## 5. Conclusion

The study demonstrates that a hydrogel containing the SHED secretome, combined with α-mangostin, significantly modulates the inflammatory response and enhances the osteogenic regeneration process in a rat model of periodontitis. This effect is mediated by a decrease in the expression of the pro-inflammatory cytokine TNF-α and a concurrent increase in the expression of the regenerative process factors BMP-2 and IL-10.

## Patient's/guardian's consent

This study did not involve human participants, and therefore, informed consent was not required.

## Ethical clearance

The research has been performed in strict accordance with the ARRIVE guidelines and guide for the care and use of laboratory animals, National Health Research and Development Ethics Standard and Guidelines Council (2017), Minister of Health, Republic of Indonesia. The research protocol has met the ethical clearances No 0.651/HRECC. FODM/V/2023 from the Research Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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