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Original Article

Orodispersible hyaluronic acid film delivery for oral wound healing in rats

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KEYWORDS
Wound healing;
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Biocompatible materials;
Re-epithelialization

Abstract *Background/purpose:* Oral wound healing undergoes a dynamic process of oral environment. This study aimed to investigate the effects of hyaluronic acid (HA) film on oral wound healing in a rat model.

Materials and methods: A total of 60 rats with tongue wounds (5 mm in diameter) were randomly divided into control ($n = 20$), HA gel ($n = 20$), and HA film groups ($n = 20$). The animals were sacrificed on either 3 or 7 days after the experiment. Clinical, histological, and quantitative reverse transcriptase-polymerase chain reaction analysis were performed to evaluate the healing rate, inflammation, re-epithelialization, and gene expression of wound healing biomarkers.

Results: The healing rates of HA gel ($84.4 \pm 9.2\%$) and HA film ($74.0 \pm 15.0\%$) were significantly higher than that of the control ($51.7 \pm 16.9\%$) on day 7 ($P < 0.001$ and $P = 0.002$, respectively). Histological analysis revealed no significant differences between the groups on day 3. On day 7, only the HA film showed significant improvement in inflammation ($P = 0.038$) and re-epithelialization ($P = 0.011$) compared to the control. Regarding wound healing biomarkers, both HA gel and HA film groups showed lower level of COL1α1 expression on day 3 compared to the control.

Conclusion: Within the limits of this study, HA film was found to be effective for oral wound healing, particularly for re-epithelialization. This finding suggests that HA film

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delivery can be beneficial not only for clinical convenience but also for promoting oral wound healing.

Introduction

Healing of oral wounds proceeds faster than that of other organs due to the mucosal tissue lining supplying blood and containing saliva, which has various growth factors, enzymes, and cytokines.[1] However, the oral environment undergoes continuous changes in pH and temperature, as well as oral flora, and the dynamic oral environment may interfere with healing.[2] Moreover, patient discomfort during oral wound healing is an important issue as the oral cavity continuously moves during pronunciation, mastication, and swallowing.[3] Thus, strategies for improving oral wound healing are of great importance in the field of dentistry.

Hyaluronic acid (HA) is a major component of the extracellular matrix (ECM) in human tissues, including oral tissues.[4] Its biological properties include moisture preservation, angiogenesis, and anti-inflammatory effects.[5,6] Clinical evidence supports that HA promotes oral wound healing. A previous study on free gingival grafts showed that HA promoted re-epithelialization of the donor sites.[7] HA was also effective in reducing the wound after labial frenectomy[8] and healing the extraction site in patients with uncontrolled type II diabetes.[9] Since HA is widely used in its gel form (e.g., Gengigel®; Ricerfarma, Milano, Italy), it can compromise drug delivery to the oral wound site due to the fluidity of saliva and volatility. Additionally, rubbing for 1–2 min to achieve the proper effect is difficult in practice.[10] Therefore, challenges remain to be addressed in the field of HA delivery.

To overcome the aforementioned concerns, an HA film that becomes orodispersible and adhesive when it comes in contact with water had been previously developed.[11] While maintaining the advantage of HA, it could provide convenience in application to clinicians and the long-lasting effect of HA on the wound sites. The aim of this study was to evaluate the effects of HA film on oral wound healing in a rat model. The null hypothesis of this study was that intraoral delivery of an HA film could not improve oral wound healing compared to HA gel or the control at the clinical, histological, and molecular levels.

Materials and methods

Preparation of HA gel and film

The HA gel was prepared using Gengigel® (Ricerfarma). 1% Gengigel® (100 μL) was transported into a syringe. The HA film was prepared according to the method of Kweon and Park.[12] HA films with a molecular weight of 0.8–1.2 MDa (Jinwoo Bio, Seoul, Korea) were prepared. A circular film with a diameter of 6 mm contained the same amount of HA as 100 ml of 1% Gengigel®. The surface of HA film and the cross-sectional side of HA film was observed using a field emission scanning electron microscope (S-4800, Hitachi, Japan) (Fig. 1).

Animal groups

This study was reviewed and approved by the Ethics in Institutional Animal Care and Use Committee of Kyung Hee Medical Center, Seoul, Korea (KHMC-IACUC-20-018) and followed the ARRIVE guidelines. The sample size was calculated using G*Power 3.1.9.7 (Heinrich Heine University, Düsseldorf, Germany). The sample size was estimated with a statistical power of 90% and a significance level of 0.05. The sample size of minimal six samples per group was calculated based on the primary outcome of a previous study.[13] A total of 60 male Sprague–Dawley rats (Youngbio, Seongnam, Korea) aged nine weeks and weighing approximately 250–300 g were used.[14,15] The rats were randomly divided into the control ($n = 20$), HA gel ($n = 20$), and HA film ($n = 20$) groups. Each group was randomly subdivided into two groups: one was the three-day sacrifice group ($n = 10$) and the other, the seven-day sacrifice group ($n = 10$).[16] In each subgroup, 7 animals were histologically analyzed and the remains were analyzed for wound healing biomarkers.

Experimental procedure

For the surgical intervention, the animals were anesthetized by administering 30 mg/kg of Zoletil 50 (Virbac Lab, Carros, France) intraperitoneally. Preoperatively, the oral cavity of the animals was cleaned with a 2% chlorhexidine solution and local anesthesia, 2% lidocaine with 1:80,000 epinephrine (Lignospan; Septodont, Saint-Maur-des-Fossés, France) was followed. To make wound uniformly, a needle of 4-0 black silk perforated the tip of the tongue and the black silk was connected to a force gauge (Wenzhou Sundoo Instruments Co., Wenzhou, China) and pulled with a constant force of 0.25 N. Thereafter, a wound was formed on the ventral surface of the tongue using a disposable biopsy punch with a diameter of 5 mm and a depth of 2 mm. After conducting compression hemostasis using a sterile cotton swab and gauze, the test materials were applied (Fig. 2). After the operation, the silk was removed and a single dose of 20,000 IU of penicillin G (Sigma–Aldrich Korea, Seoul, Korea) was administered intramuscularly in all animals. After 3 and 7 days, the animals were euthanized under anesthesia.

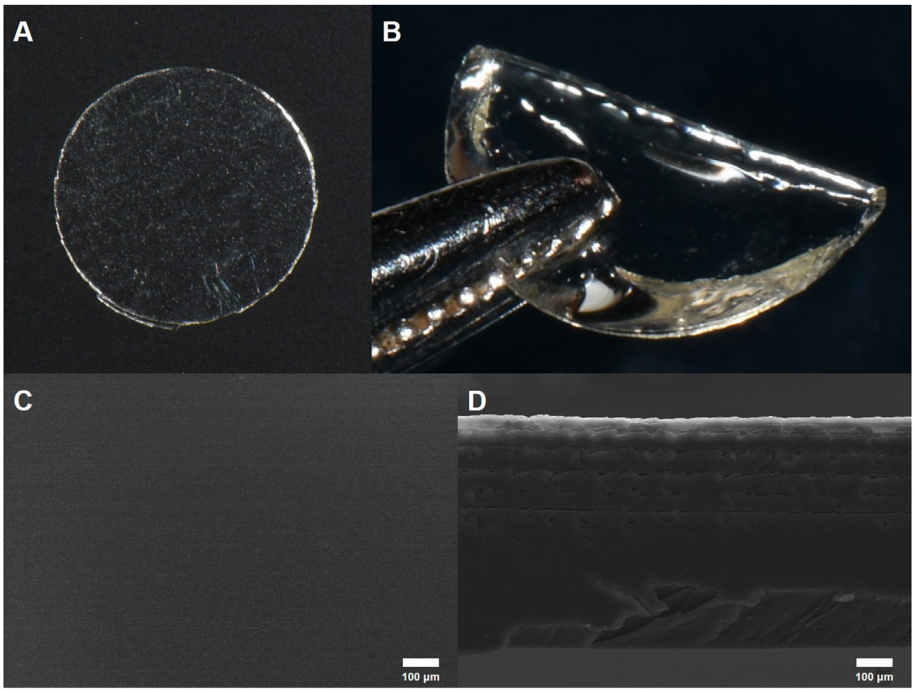

Figure 1 The prepared hyaluronic acid (HA) film. (A) HA film, (B) Water-soaked HA film showed viscous property. (C) Surface view of HA film (scanning electron microscope image, x1000), (D) Cross-sectional view of HA film (scanning electron microscope image, x1000).

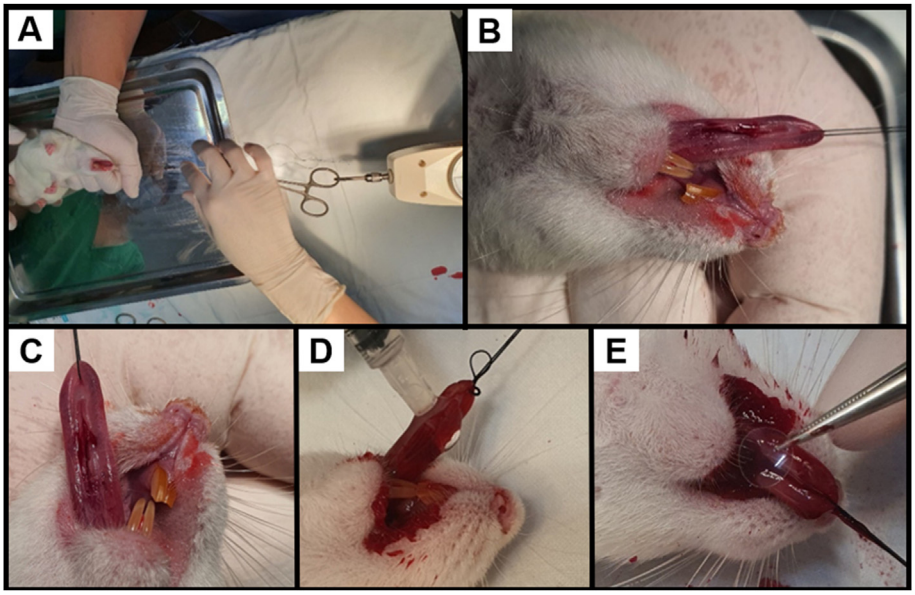

Figure 2 Experimental procedure of oral wound formation and HA application. (A) 4-0 black silk was connected to the tongue and pulled with a force gauge of 0.25 N. (B) Hemostasis of a wound with a diameter of 5 mm using sterile cotton swabs and gauze. (C) Control group: No applications after hemostasis. (D) HA gel group: 100 μL of 1% HA gel was applied. (E) HA film group: 100 μL of 1 circular HA film with a diameter of 6 mm was attached.

Clinical healing analysis

For clinical analysis, the healing rate (%) was measured by calculating the wound healing area (Fig. 3). To correct the magnification, the specimen was placed on a grid paper of 1 mm length and the image was obtained. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the area, and after obtaining the pixel value of a circle with a diameter of 5 mm, healing rate was calculated using the pixel value of the wound, as in the following formula:

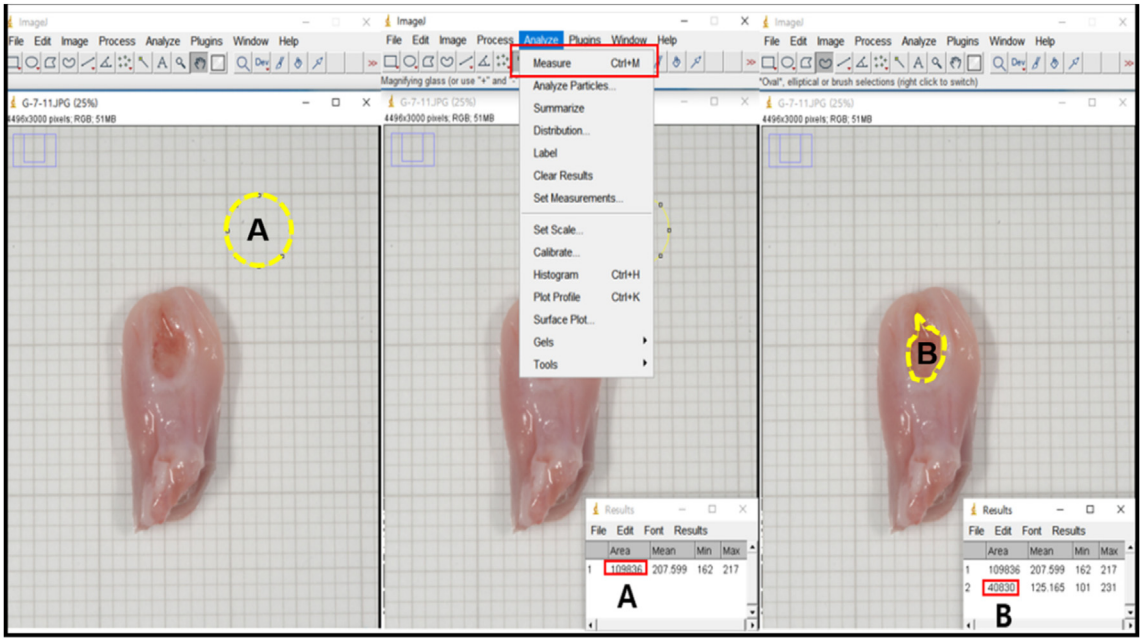

Figure 3 Measuring wound healing rates with ImageJ software. A, Pixel value of a 5 mm diameter circle; B, Pixel value of the wound surface.

$$\text{Healing rate} = \frac{A - B}{A} \times 100(\%)$$

Here, A refers to pixel value of circle with diameter of 5 mm and B refers to pixel value of remaining wound.

Histological analysis

After euthanizing, the tongue was separated and fixed in 10% formalin solution. The specimens were vertically cut to the wound site and the tongue. Each section was stained with hematoxylin and eosin and observed using a digital slide scanner (Motic, Kowloon Bay, Hong Kong). The degree of inflammation and re-epithelialization were observed in each group by two examiners. Histological examination was performed as described by Rahman et al. and Özay et al., with some modifications.[17,18] Inflammation scores were defined by the presence of inflammation related cells. The inflammation score was 0 when there were no signs of inflammation, 1 when giant cells, lymphocytes, and plasma cells were shown, 2 when neutrophils and cells in the presence of inflammation related celes shown in the score 1, and 3 when microabscess formed.

Re-epithelialization score was defined as follows: 0 when the absence of epithelial proliferation was seen in more than 70% of the tissue (≥3.5 mm), 1 when incomplete epidermal organization was seen in more than 50% of the tissue (≥2.5 mm), 2 when epithelial proliferation was seen in more than 50% of the wound site (≥2.5 mm), and 3 when complete epidermal remodeling was observed (Table 1).

Table 1 Scores for analysis in histological examination.

Scores	Inflammation	Re-epithelialization
0	No inflammation	Absence of epithelial proliferation in ≥70% of tissue
1	Presence of giant cells, lymphocytes, plasma cells	Incomplete epidermal organization in ≥50% of tissue
2	Presence of neutrophils and cells in score 1	Moderate epithelial proliferation in ≥50% of tissue
3	Presence of microabscess formation	Complete epidermal remodeling

Quantitative real-time polymerase chain reaction (qRT-PCR)

The integrity and concentration of the RNA were evaluated using the StepOnePlus Real-Time PCR system and measured by SYBR green fluorescence using a commercial reagent (Power SYBR Green Master Mix; Thermo Fisher Scientific, Cleveland, OH, USA). Total RNA was isolated from the samples ($n = 3$ per group). Briefly, 2000 μg of total RNA was converted into complementary DNA (cDNA) using reverse transcriptase (SuperScriptII Reverse Transcriptase; Invitrogen, Carlsbad, CA, USA). The cycling conditions were as follows: denaturation for 15 min at 95 °C, followed by 40 amplification cycles of denaturation for 15 s at 95 °C, and annealing for 30 s at 59 °C. The cDNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels using the $2^{-\Delta\Delta Ct}$ method.[19] The experiments were performed in triplicate. Primer sequences used for PCR are listed in Table 2.

Table 2 Primers used for quantitative real time polymerase chain reaction analysis.

Gene	Forward Primer sequence (5′–3′)	Reverse Primer sequence (5′–3′)
COL1α1	GCCCTGGATACCAACTACTG	GAGGAGCAGGGACTTCTTGA
TGF-β1	ATGAGGTCTACTCGGCAAAC	CCAGGTCCTTCCTAAAGTCA
GAPDH	ACAGTCAAGGCTGAGAATGG	GATCTCGCTCCTGGAAGATG

Abbreviations: *COL1α1*, Alpha 1 type I collagen; *TGF-β1*, Transforming growth factor beta 1; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Data were analyzed using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA). The clinical and histological results are presented as the mean ± standard deviation. After normality test using the Kolmogorov–Smirnov test, data from the clinical, histological, and qRT-PCR analysis was statistically analyzed with the Kruskal–Wallis test ($P < 0.05$). Post-hoc analysis was performed using the Mann–Whitney test ($P < 0.05$).

To assess inter-observer reliability in the histological analysis, we calculated the intra-class coefficient values between the two examiners. The intraclass coefficient value was highly reliable, with an inflammation score of 0.905 and a re-epithelialization score of 0.915.

Results

Clinical healing analysis

The difference in healing rate between the control, HA gel, and HA film groups was not statistically significant on day 3 ($P = 0.172$). The healing rate of the HA gel (84.4 ± 9.2%) and the HA film groups (74.0 ± 15.0%) was higher than that of the control group (51.7 ± 16.9%) on day 7 ($P < 0.001$ and $P = 0.002$, respectively). However, the difference in healing rate between the HA gel and the HA film groups was not statistically significant on day 7 (Fig. 4).

Histological analysis

There were no significant differences in inflammation ($P = 0.938$) and re-epithelialization ($P = 0.849$) between the control, HA gel, and HA film groups on day 3. Inflammatory cells, such as macrophages, were found in all groups on day 3 (Fig. 5).

On day 7, the inflammation and re-epithelialization scores of the HA film group were better than those of the control group ($P = 0.038$ and $P = 0.011$, respectively). However, the difference in inflammation score ($P = 0.710$) and re-epithelialization score ($P = 0.165$) between the control and the HA gel group were not statistically significant. Normal connective tissue and distinct rete pegs were observed in the HA film group.

qRT-PCR

The expression levels of alpha 1 type I collagen (COL1α1) and transforming growth factor beta 1 (TGF-β1) were lower in both HA gel and HA film groups than in the control group on day 3; however, only COL1α1 expression showed statistical significance ($P < 0.05$). Conversely, the amounts of

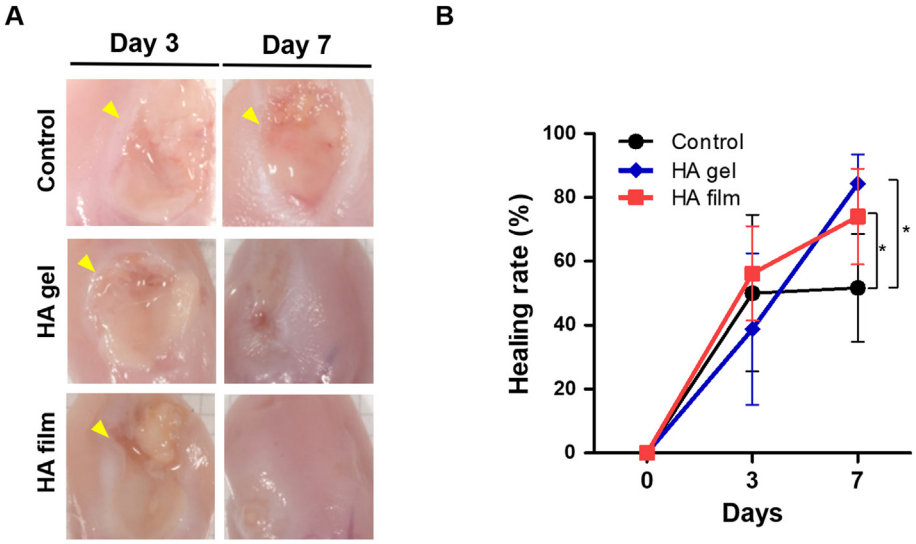

Figure 4 Clinical healing rate analysis. (A) Clinical view of ventral surface of the tongue on day 3 and day 7. The wound margins are indicated with arrows. (B) Healing rates of the groups. Post-hoc analysis was performed using the Mann–Whitney test. $P < 0.05$ was considered statistically significant.

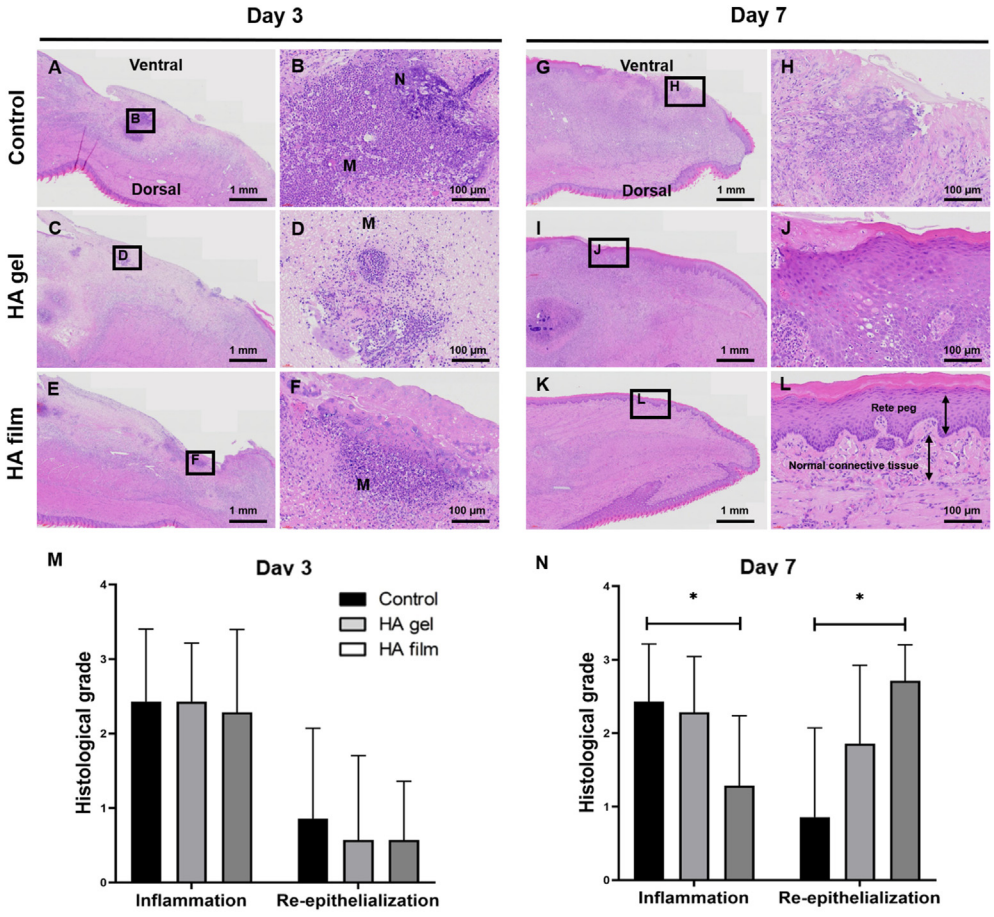

Figure 5 Histological analysis of the wounds on day 3 and 7 day after treatment, stained with hematoxylin and eosin. (A, B, G, H) Control group, (C, D, I, J) HA gel group, and (E, F, K, L) HA film group. (M, N) Histological evaluated grade expressed as the mean ± standard deviation in inflammation and re-epithelialization, ($P < 0.05$), M: Macrophage, N: Neutrophil. Scale bars: 1 mm, 100 μm.

COL1α1 and TGF-β1 were higher in the HA gel and HA film groups than in the control group on day 7, but this difference was not statistically significant (Fig. 6).

Discussion

There are many products derived from HA for wound dressing because of their biocompatibility, biodegradability, hydrophilic character, and fewer side effects.[20,21] The beneficial effects of HA are closely related to angiogenesis, inflammation, and tissue regeneration.[5] Previous studies have confirmed that HA application improves wound healing in dental sockets after tooth extraction[22] and bone healing after xenografts.[23] Despite the fact that HA can facilitate oral wound healing, the delivery of HA to the moving tissues in the oral cavity remains a challenge.

According to our study, both HA delivery groups significantly promoted wound healing clinically compared to the control group on day 7. These findings are consistent with the results of a study that reported that HA improved oral wound healing.[24,25] However, there were no significant differences in the promotion of wound healing between the HA gel and HA film groups in this study.

Inflammation is an important stage during the healing process. This event attracts macrophages and neutrophils to the wound tissue and facilitates healing by releasing cytokines and a variety of signals.[26] However, delayed inflammation prolongs wound healing by the formation of toxic free radicals and reactive oxygen species and increases the risk of additional infection. In this study, the level of inflammation on day 3 in all groups was similar. This is probably a required part of the normal healing process.[2,27] However, the inflammation level in the HA film group was significantly reduced on day 7. Small-size HAs (6–20 kDa) are known to accelerate angiogenesis and pro-inflammatory response.[28,29]

HA delivery groups presented a significantly better quality of re-epithelialization on day 7; however, significant difference on re-epithelialization was found between HA film and the control. Re-epithelialization of a wound, the resurfacing of a tissue with new epithelium starts approximately after 1.5–2 days and lasts until the third stage of wound healing.[30] This is closely related to the adequate migration of keratinocytes.[31] For this process, smooth intra-signaling with dermal fibroblasts and ECM with related cytokines present in the wound fluid is needed. HA is a good matrix that provides a structural scaffold and maintains

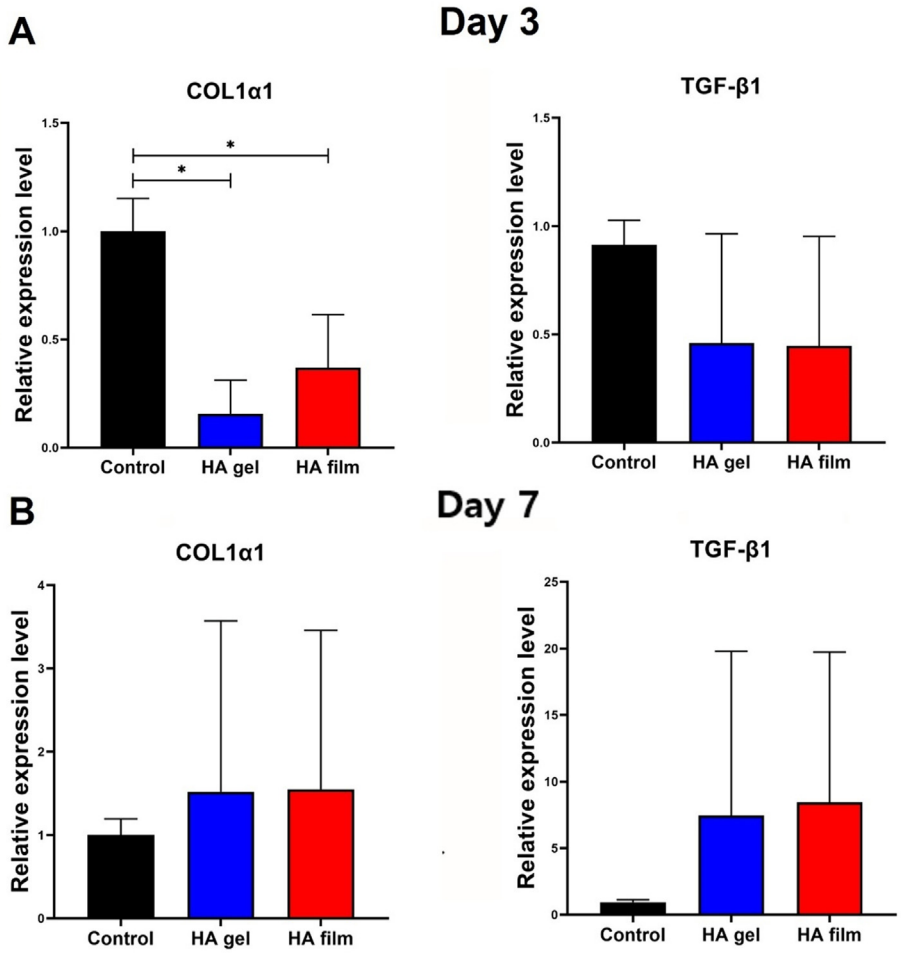

Figure 6 Gene expression of wound healing biomarkers by qRT-PCR.(a) The expression of COL1α1 was statistically significant among the groups on day 3 ($P < 0.05$). (B) There were no significant differences in the biomarkers among the groups on day 7. COL1α1, Alpha 1 type I collagen; TGF-β1, Transforming growth factor beta 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

vascularization to increase the movement of growth factors, key cells, and cytokines.[32]

Regarding inflammation and re-epithelialization on day 7, both HA groups showed better results than the control groups. However, only the film group showed a statistical significance. This difference could be attributed to the characteristics of the HA films. The orodispersible and adhesive character of the HA film provided stability in the tongue tissues and facilitated the biological effects of HA. A low-molecular-weight HA film has porous structures that induce cell migration related to wound healing.[33]

Collagen, the key component of ECM, regulates the elasticity and force of the tissue and is also related to scar tissue.[34] Excessive expression of collagen type 1 results in hypertrophic scar development and irregular structure formation in the tissue.[35] In other words, hypertrophic scar is the result of excessive collagen synthesis and ECM protein accumulation during the wound healing process.[36] HA is known to inhibits collagen synthesis and fibroblast synthesis by blocking the initial expression of TGF-β1.[37] As a result, it prevents the formation of scars caused by excessive collagen synthesis and increases the moisture and oxygen permeability of the wound surface. COL1α1 is a major component of the soft tissue ECM and is a known wound-healing gene.[38] TGF-β1, a cytokine, is involved in the wound healing process which promotes fibroblast proliferation and collagen synthesis.[39] Excessive expression of TGF-β1 causes excessive synthesis of collagen and fibroblasts and induces the expression of connexin 43, which promotes hypertrophic scar.[40,41] The results obtained on day 3 confirmed that the expression of COL1α1 tended to be less in the HA gel group or the HA film group than in the control group. However, there was no statistically significant difference in TGF-β1 expression. This confirmed our hypothesis that HA prevents excessive collagen synthesis and formation of fibroblast proliferation in the early stages of wound healing, and increases its interaction with wound healing cytokines, proteins, and healing signals.

This study showed better quality results by applying HA in its film form for oral wound healing. Compared to conventional gel-type formulations, HA film is easier to apply to oral wounds and is clinically convenient because of its orodispersible and adhesive properties. Furthermore, it has an additional advantage over conventional HA gel in that special application methods or additional procedures are not needed, owing to its adhesive and long-lasting features.

However, the present study also has some limitations. First, since the tongue is a moving organ, the punched tissue could be further damaged while chewing, swallowing, and scratching with food. Further studies conducted in other non-moving areas, such as the palate, are needed. Second, although the information on how long HA film and gel remain on the tissue after application would be clinically relevant, it was not explored in the present study. Future studies are needed to evaluate the difference in the duration of HA film and HA gel.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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