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Potency of hyaluronic acid from eggshell–membrane for open gingival embrasure reconstruction following orthodontic tooth movement (a histomorphological study)

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ABSTRACT

Background: The aim of this research was to assess the effectiveness of eggshell–membrane (ESM)-containing hyaluronic acid (HA) in the treatment of open gingival embrasure (OGE) following orthodontic tooth movement (OTM).

Materials and Methods: This study is an *in vivo* quasi experimental research. A total of 24 *Cavia cobaya* were equally divided into two groups, treatment (10% HA injection) and control (phosphate-buffered saline [PBS]). A separator was inserted between mandibular incisors to induce an OGE. A volume of 20 μl of either PBS (n = 12) or ESM extract (n = 12) was locally injected within the interdental papilla. Decapitation of animals was made on day 1, 4, and 7 postinjection. The staining was done using hemotoxylin and eosin to observe angiogenesis and Mallory to observe the collagen density. Fourier-transform infrared spectroscopy (FTIR) and thin-layer chromatography (TLC) analysis were performed to detect the amount of HA available in ESM. The results were then compared with independent t-tests and the Mann–Whitney test. The level of statistical significance was set at 0.05.

Results: The FTIR and TLC analysis showed that HA was successfully identified in the ESM samples. Local injection of 10% HA induced an increase of angiogenesis compared to the control group on day 1 and 4 postinjection ($P < 0.05$). Significant differences ($P < 0.05$) were also noted in the collagen density and the growth of interdental papilla on day 4 and 7 postinjection.

Conclusion: ESM has the potential effect of regenerating the interdental papilla construction after OTM by increasing the collagen fiber density and inducing angiogenesis.

Key Words: Hyaluronic acid, open gingival embrasure, orthodontic tooth movement

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INTRODUCTION

The goals of orthodontic treatment are corrected teeth position and alignment, reduce occlusal trauma, and remodeling of surrounding tissue, including periodontal ligament, alveolar bone, and gingiva, indicates a good adaptation against the orthodontic force applied.[1,2] An unexpected condition that occurs following orthodontic treatment is opening gingival embrasures (OGE) or "Black Triangle." OGE is defined as the loss ability of interdental papillae (interproximal tissue) to fill up the embrasures beneath the orthodontic contact point, resulting in the appearance of a triangular area (black triangle) which

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affect smile esthetics and leads to several functional problems such as food impaction and phonetic problems.[3,4] OGE rate following orthodontic tooth movement (OTM) is still high. Tanaka *et al.* reported that OGE affects 43.7% of adult orthodontic patients.[5] Several surgical techniques have been developed to improve OGE; however, their adverse effects have limited an extensive application.[4] Gingival grafting procedures are often selected; however, the success rate is low due to the gingival tissue minimal blood supply.[6]

The use of hyaluronic acid (HA) has been claimed to be an effective treatment for OGE. Mansouri *et al*. described that HA gel injections used to reconstruct the interdental papilla showed more than 50% of development in the samples.[7] HA is attached to the surface receptor CD_{44}, a proteoglycan with heparin sulfate that regulates migration and cells proliferation of the cell to cell and cell-matrix adhesion.[8] Recently, natural materials are being used and developed massively.[9] Previous reports mentioned that HA could be obtained from the natural eggshell–membrane (ESM). Interestingly, the composition of HA in ESM is at the highest level compared to other sources, reach 0.5%–10% of the total weight.[10] Therefore, the aims of this study are to develop and investigate the effect of HA from ESM in the improvement of OGE following OTM.

MATERIALS AND METHODS

Preparation of hyaluronic acid from eggshell–membrane

This study is an *in vivo* experimental research. From 8 kg of chicken eggshell waste, we extracted 105.3 g of ESM. A portion of the ESM extract was analyzed using thin-layer chromatography (TLC). The functional group formation of HA contained in ESM was determined using Fourier-transform infrared spectroscopy (FTIR) at a wave number ranging from 500 to 4000/cm, which was operated following the protocol described by Alhasyimi *et al.*[11]

Animal experiments

Ethical approval for this study (approval clearance number 001437/KKEP/FKG-UGM/EC/2018) was provided by the Research Ethics Committee of the Faculty of Dentistry, UGM. Twenty-four 14-week-old male guinea pigs weighing ±350 g were included in the study. All the animals were randomly divided into two groups, the control group (n = 12) received phosphate-buffered saline (PBS) injection and the treatment group (n = 12) received 10% ESM injection. Both groups we divided into three small subgroups based on the repetition of applications (1, 2, or 3 times) as shown in Table 1. All the animals were anesthetized with a mixture of ketamine (Kepro™, the Netherlands) and xylazine (Xyla™, the Netherlands) at doses of 25 and 5 mg/kg body weight injected intraperitoneally. To adopt a condition that resembles OGE, an elastic separator (American Orthodontics, USA) was inserted between the animal's mandibular incisors to move the teeth laterally to induce a space opening between the mandibular incisors. An undercut notch was made using a low-speed fissure bur >2 mm from the level of the interdental papilla crest [Figure 1]. Notch-papilla crest distance (NPD) was used as endpoint references, and notch-incisal distances (NID) as the initial point, the differences between NID and NPD were then used to calculate the growth of the interdental papilla (GIP)

Figure 1: Design of experimental orthodontic movement to represent opening gingival embrasure model in the guinea pig, (A) elastics Power-O; (B) Undercut notch.

Table 1: Groups of experiment

Label	Group
C1	Control group; repetition of application 1 time (1 day postinjection)
C2	Control group; repetition of application 2 times (4 days postinjection)
C3	Control group; repetition of application 3 times (7 days postinjection)
T1	Treatment group; repetition of application 1 time (1 day postinjection)
T2	Treatment group; repetition of application 2 times (4 days postinjection)
T3	Treatment group; repetition of application 3 times (7 days postinjection)

which constituted the interdental papilla growth to covered up the OGE [Figure 2].

After 7 days of space opening, 20 µl of either PBS or ESM extract was locally injected within the interdental papilla, approximately 5 mm apical from the interdental papilla crest, using a 31G ultrafine needle (BD Ultra-Fine™, USA). A second application was performed on day 3 (C2, C3, T2, and T3) and the third repetition on day 6 (C3 and T3). The evaluation of the NPD was done using a digital sliding caliper (Pro-Max®, China).[12] The evaluation was carried out on days 1, 4, and 7 postinjection. All the measurements were conducted by the same experienced researchers and repeated thrice. The mean of these measurements was used as the representative score for each group.

Histological preparation

The tissues were decalcified using 10% ethylenediaminetetraacetic acid for 2 weeks. Each sample was then embedded in paraffin wax for 12–16 h and sectioned mesiodistally parallel to the long axis of the animal incisor. Specifically, 5-µm thick serial sections were cut at 50-µm intervals with a microtome blade. Tissue paraffin sections were then stained using hemotoxylin and eosin, to highlight the number of new blood vessels (angiogenesis), and Mallory staining to observe the collagen fiber density.

Histological observation

A single, blinded experienced researcher performed the histological evaluation. Three histological slices from each group were examined, and six areas were randomly selected as regions of interest (ROIs) that extended vertically of the mesial surface's incisor alveolar bone. Data were obtained from the 6 ROIs using an optical light microscope with a mounted digital camera (Olympus GmbH, Hamburg, Germany) under ×400 magnification. The angiogenesis count per field was calculated using ImageJ® software (National Institutes of Health, Bethesda, Maryland, USA). Scoring for determination of the collagen fiber density follows a protocol outlined by Tandelilin *et al.*, showing a low (Score 1), medium (Score 2), and high range of collagen density (Score 3).[13]

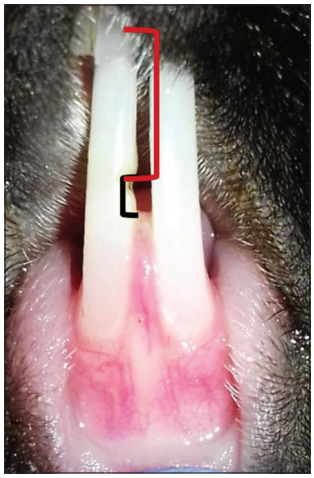

Figure 2: Schematic figure determination of growth of the interdental papilla. Notch-papilla crest distance (black line) was used as endpoint references, and notch-incisal distances (red line) as the initial point, the differences between notch-incisal distance and notch-papilla crest distance were then used to calculate the growth of the interdental papilla.

Statistical analysis

The GIP and angiogenesis data were analyzed by an independent *t*-test. The Mann–Whitney test was utilized used to analyze the density of collagen fibers. Values of $P < 0.05$ were considered statistically significant. All of the data were analyzed using the IBM SPSS software 22.0 (International Business Machines Corporation, New York, USA).

RESULTS

Characterization of material

Confirmatory tests using TLC showed that HA was detected from ESM under ultraviolet light at 254 nm. Furthermore, functional groups HA were also identified from the ESM samples using FTIR that indicated the adsorption peak wavenumber of HA functional groups (carboxyl, hydroxyl, and ether).

In vivo study

All the experimental procedures were well tolerated by the animals, generally. Our results showed that the mean value of the treatment group was found to be higher than the control group [Table 2]. Interestingly, the T2 group presents the highest mean value of the treatment, whereas the highest mean value of the control group was in the C3 group. The value of GIP represented the difference in the value of NPD and NID measured at days 1, 4, and 7 postinjection. The results of the normality test showed that the significance values of both groups were normally distributed. The Levene's test for homogeneity indicates that the data were homogeneous. The results of the normality and homogeneity test show that the data can be subjected to parametric analysis test through the independent *t*-test. The analysis results showed that the T2 group has significant ($P < 0.05$) highest GIP value compared with all the experimental groups.

Table 2: Comparison of growth of the interdental papilla, collagen fiber density, and angiogenesis at each observation time point between 2 groups tested[a]

Parameters	Control group	ESM group	*P*
GIP			
Day 1	2.423±0.433	2.901±0.575	0.092
Day 4	4.473±0.701	5.792±0.841	0.048*
Day 7	4.814±0.964	5.503±0.716	0.036*
Collagen density			
Day 1	0.933±0.098	1.003±0.121	0.091
Day 4	1.683±0.169	2.117±0.187	0.022*
Day 7	2.049±0.151	2.425±0.113	0.048*
Angiogenesis			
Day 1	4.833±0.663	7.033±0.362	0.033*
Day 4	5.733±0.438	9.986±0.843	0.019*
Day 7	5.691±0.858	6.051±0.563	0.083

[a]Tested by independent *t*-test for GIP and angiogenesis; tested by the Mann-Whitney test for collagen density. Values are presented as a mean±SD. *$P<0.05$, significant differences between the two groups. SD: Standard deviation, GIP: Growth of the interdental papilla, ESM: Eggshell-membrane

Considering the histological observation, it has been known that both angiogenesis and collagen density were showed a trend toward an increase in groups treated with ESM compared to the control group [Figure 3]. The sum of angiogenesis and observation of collagen fiber in both groups is presented in Table 2. The highest total count of angiogenesis presented by Group T2 which have relatively high significant ($P < 0.05$) different compared with all the groups, whereas the total count of collagen obtained by semi-quantitative technique and indicated that the highest value presented by Group T3 and this values were statistically significant ($P < 0.05$) on day 7 postinjection [Table 2]. Following the injection of HA from ESM, the inner granules containing HA were identified within the submucosal layer without indication of foreign body cells reaction or inflammation sign. The inner granules gradually disappear from day 4 to 7. These results suggest that ESM has the potential effect to regenerate the interdental papilla construction after OTM by increasing the collagen fiber density and inducing angiogenesis in the guinea pig.

DISCUSSION

The OGE may occur following orthodontics tooth movement due to tissue injury caused by stretched gingival fiber, loss of attachment, change of papillae position, and alveolar bone height reduction.[5] To overcome these conditions, this *in vivo* study was conducted to investigate the effect of new biomaterial for tissue reconstruction and regeneration therapy in the case of OGE. The obtained data indicate that groups treated with ESM-containing HA injection have the potential to stimulate angiogenesis and inducing collagen fiber formation. ESMs are known to have various bioactive compounds, including collagen, protein, and glycoprotein, as well as organic components such as glycosaminoglycan (GAG) with different structures, including chondroitin sulfate, HA, and heparan sulfate.[14] In the previous study, numerous biocompatibility analyses, such as cytotoxicity test, acute toxicity test, hemocompatibility test, and oral mucous membrane irritation test have been done, and the results also showed that ESM has very excellent biocompatibility and biodegradability and it is comparable to collagen type I as well.[15]

ESM contains the highest level of HA reaches 0.5%–10% of ESM total weight, which is a high level compared with other sources such as synovial liquid, and chicken comb.[16] HA belongs to a group of a polysaccharide called (GAG) which play important roles in healing properties of periodontal tissue. The HA tissue regeneration abilities are related to its interaction with the surface receptor CD_{44} which acts as the hyaluronan receptor for signaling, stimulating cell migration, and proliferation in addition to maintaining the formation of collagen fibers and extracellular matrix in connective tissue.[17] Collagen plays a central role in the tissue regeneration process as it provides a structural framework and elasticity for the regenerating tissue.[18] The result of this study showed that groups treated with HA have a higher score of collagen density and angiogenesis, and the repetitive doses increase the collagen and angiogenesis formed. It has been known that HA interaction with CD_{44} receptors leads to an acceleration of several cell activity including fibroblast to produce collagen fibers by the stimulation of fibroblasts growth factor.[19] HA proved to possess a protective effect on collagen synthesis as well.[20] In addition, HA also inhibits transforming growth factor-beta-1 and reduce the inflammatory response. These actions result in faster tissue healing with less scar formation. This enhanced regeneration activity is attributed to increased collagen formation and angiogenesis.[21]

Angiogenesis is an essential part of the proliferation phase of the healing process, it represents an outgrowth of new blood vessels from existing ones to provide nourishment to newly forming tissues, improves cell survival, and contributes toward natural

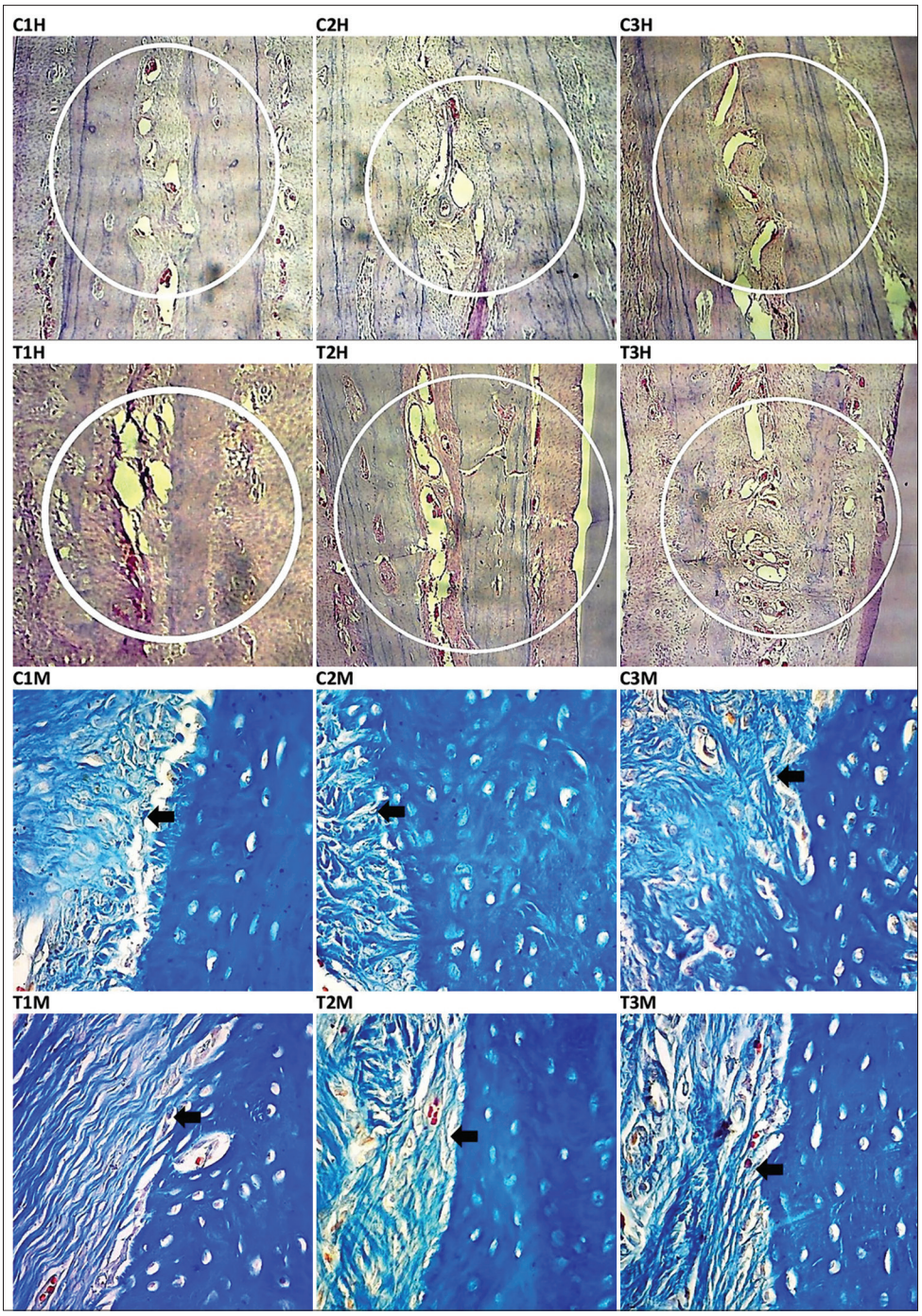

Figure 3: The figure of histological lumens of new blood vessels (angiogenesis, circled) with haemotoxylin-eosin staining and collagen density (black arrows) in both of groups.(×40 for angiogenesis; ×400 for collagen). H indicates hemotoxylin-eosin-staining; M, Mallory-staining.

immunity as well. An accelerated improvement of blood capillaries that provisionally filled damaged sites are crucial to tissue remodeling and repair.[22,23] HA, known to have pro-angiogenic properties, the engagement of HA toward surface receptors CD_{44} induces the endogenous release of vascular endothelial growth factor which, in turn, stimulates endothelial cells proliferation, blood vessel growth, and build-up structures of new capillaries as well.[23] Interestingly, the highest total count of angiogenesis was present in Group T2 rather than Group T3. Originally, we thought that the increasing doses of HA given would increase the total count of angiogenesis. This finding aligns with a previous report by Ozgenel and Etöz,[24] that mentions that highly concentrated HA in tissue inhibits the motility and phagocytosis capacity of

granulocyte which affects the debridement process in initial phases of the healing process.

Clinical observation in OGE groups treated with HA showed that papillae become firmer and have a convex shape. This suggests the presence of tissue regeneration [Figure 4]. Furthermore, the augmentation of the interdental papillae covering the OGE was found in both groups. The closures of OGE in treatment groups appeared at day 4 postinjection while in the control groups appeared at day 7 postinjection when the regeneration process was almost done. One of the key esthetic factor in the smile of an individual is considered by the presence of the interdental papilla. Some condition like a black triangle that can change in the values of the width/height ratio of interdental papilla can even ruin the appearance. Regeneration or rejuvenation of interdental papilla can be done by increasing the collagen fiber density and inducing angiogenesis. In this regards, previous studies by Pi *et al.*[25] suggest that HA plays an important role in maintaining the structure and promoting the protection of the extracellular matrix and collagen. HA form layers that determine the level of adhesion, migration, and shape of the cell-attached and populating those layers.[26] Interestingly, the comparison based on the multiple applications showed that the highest value of GIP was carried out by Group T2. The accumulation of HA in high concentration leads to an inhibition of granulocytes, which affects the migration, cleaning, and healing of the injured sites. Meanwhile, using a single dose, the therapeutic effect of HA could not reach the maximum level.[27]

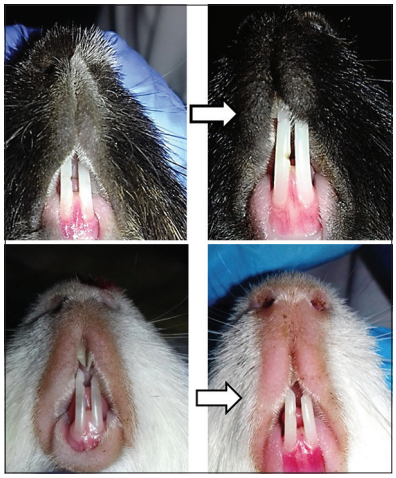

Figure 4: Open gingival embrasure initial and final observation in both groups tested. Papillae become more firm and acquire a convex shape; This phenomenon was seen more often in groups treated with eggshell–membrane injection (upper) compare with the control group (lower), indicating the occurrence of tissue regeneration.

CONCLUSION

ESM containing HA has the potential effect to enhance the regeneration process of OGE following OTM. A double dose of ESM injection can be an effective method for enhancing the growth of interdental papilla in the guinea pig. However, further studies are required to validate the probable efficacy of ESM in a clinical study.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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