Development of eggshell membrane as an alternative for black triangle reconstruction following orthodontic tooth movement

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Abstract. Improper tissue alterations can occur following orthodontic treatment. One of such alterations is the opening of the interdental papilla, the so-called "black triangle" (BT), which can affect smile appearance and lead to functional problems. Use of hyaluronic acid (HA) to treat BT is an innovation that has significant results. The newest sources of HA can be extracted from the chicken eggshell membrane (ESM), which might have the potential to treat BT as effectively as commercial HA. The aim of this study was to investigate the effectiveness of ESM containing HA in treating BT. Thin-layer chromatography (TLC) was used to detect HA isolated from ESM, while Fourier-transform infrared spectroscopy (FTIR) analysis was performed to confirm the formation of HA. A total of 18 Cavia cobaya were equally divided into two groups, treatment (10% HA injection) and control (phosphate-buffered saline), to confirm the efficacy of the materials in vivo. Parameters obtained from clinical observations of the differences between ligature-papilla distances (LPD) and socket-papilla distances (SPD) measurement was compared with independent t-tests. The FTIR analysis showed that HA was successfully identified from the ESM samples. This was indicated by the adsorption –OH band at 3419 cm⁻¹, the –COOH band at 1634 cm⁻¹, and the C–O–C saccharide unit at 1049 cm⁻¹. Moreover, HA was also detected from ESM under UV light at 254 nm using thin layer chromatography (TLC) analysis. Local injection of 10% HA induced an augmentation effect of the interdental papilla compared to that in the control group (P< .05). The results of this study reveal that ESM containing HA has the potential effect to regenerate the interdental papilla construction after orthodontic tooth movement.

1. Introduction
Orthodontic treatment is important to improve appropriate occlusion and achieve an optimum dentofacial function [1-2]. Open gingival embrasures or interdental papilla may occur after orthodontic treatment. Open gingival embrasures, also known as “black triangles” (BT), refer to the empty spaces below the interproximal contact caused by the lack of interdental gingival papilla [3]. The incidence of BT following orthodontic treatment is still high and has been a controversial issue to be debated. Ko-Kimura et.al reported a relatively high incidence of 41.2%–66.7% of BT [4]. Black triangles may occur due to various factors such as aging, loss of height of the alveolar bone, conditions that occur during orthodontic treatment such as stretched gingival fiber, attachment loss resulting in gingival recession, a tight contact near the incisal edge, excessively divergent roots (may occur naturally or as an effect of orthodontic treatment), and other alterations resulting from long-term
orthodontic treatment [5]. Although various modalities have been introduced to improve BT, the adverse effects and the complications of the clinical operation have limited their extensive application.

Hyaluronic acid (HA) is a biomaterial widely used in biomedical applications, as well as an essential component of periodontal tissues, which has various structural and physiological functions [6][7]. Use of HA to treat BT is an innovation that has significant results, and the success rate is about 50% within 6 months after HA application [7]. Interestingly, HA can be found in any extracellular matrix and can be extracted from animal or bacterial sources through fermentation process [8]. Chicken eggshell membrane (ESM) contains HA with a composition of about 5%–10% of the total weight, which is the highest level compared with any other sources; thus, this material can be a potential source of HA [9]. Therefore, the aim of this study was to develop and validate the effects of ESM containing HA on the augmentation of the interdental papilla.

2. Materials and Methods

2.1. Preparation of Hyaluronic acid from chicken eggshell membranes

ESM weighing 105.3 g retrieved from about 8 kg of chicken eggshell waste was extracted using 70% ethanol as a solvent, which resulted in 1 g of ESM extract in the form of a sticky powder. A portion of the ESM extract was analyzed using thin-layer chromatography (TLC) to separate the bioactive compound HA using a silica gel GF254-coated glass plate as the stationary phase and eluent which is n-butanol:glacial acetic acid:Aquadest (BAA) at a ratio 4:1:5 and n-butanol:ethanol: Aquadest (BEA) at a ratio 4:1:2.2 as the motion phase. HA, being a polar compound interacts strongly with silica gel and moves along the motion phase leaving stains that can be detected using UV light (λ1: 254 nm; λ2: 366 nm). Afterward, the functional group formation of HA isolated from ESM was determined by Fourier-transform infrared spectroscopy (FTIR) (Thermo Scientific Nicolet iS10, USA) at a wave number ranging from 500 to 4000 cm⁻¹, which was performed following the protocol outlined by Alhasyimi et al. [10].

2.2. Animal experiments.

Animal experiments were conducted to validate the effectiveness of the material for tissue reconstruction in vivo. Ethical permission was obtained from the Research Ethics Committee of the Faculty of Dentistry, UGM, with the approved clearance number 001344/KKEP/FGK-UGM/EC/2018. A total of 18 male guinea pigs (Cavia cobaya) aged 14 weeks and weighing approximately 350 g were used in this study. They were maintained in an appropriate breeding room with a 12/12-h light–dark cycle at 25°C and a humidity range of 64–80°F. During the experiments, the animals were administered a diet of standard laboratory pellets and tap water ad libitum. All the animals were randomly divided into two groups, the control group (n = 9) that received phosphate-buffered saline (PBS) injection and the treatment group (n = 9) that received 10% ESM injection. All the animals were anesthetized by an intramuscular injection of a mixture of ketamine hydrochloride (Kepro™, the Netherlands) and xylazine (Xyla™, the Netherlands) at doses of 25 mg/kg and 5 mg/kg body weight, respectively.

To mimic a condition that resembles an open interdental gingival embrasure, an elastic separator (American Orthodontics, USA) was inserted between the animal’s mandibular incisors to move the teeth laterally that can induce a space opening between the mandibular incisors (Figure 1). An undercut notch was made using a low-speed fissure bur above 2 mm from the level of the interdental papilla crest (Fig. 1). This procedure reinforced the retention of the ligature spring that was used as endpoint references (ligature-papilla distance/LPD). As an initial point, socket-papilla crest distances
(SPD) was also determined, and the differences between SPD and LPD was then used to calculate the augmentation of interdental papilla (AIP) which constituted the interdental papilla growth.

After 7 days of space opening, ESM containing HA was injected approximately 5 mm apical from the interdental papilla crest, using a 31 G ultrafine needle (BD Ultra-Fine™, USA), once a day. The evaluation was done through the examination of the ligature–papilla interdental crest distance (LPD) using a clinical photograph and a digital sliding caliper (Pro-Max®, China) with a minimum measurable distance of 0.01 mm [11-12]. The evaluation was carried out on day 2 post-injection (T1). The evaluation results were compared to those of the initial examination (T0) before injection to determine whether there are significant changes in post-treatment for both the groups. All the measurements were conducted by the same experienced researchers and repeated three times. The mean of these measurements was used as the representative value for each distance. The data were then analyzed by an independent t-test using the Statistical Package for the Social Sciences version 22.0 (SPSS 22.0, USA) software.

![Figure 1. Design of experimental orthodontic tooth movement to represent opening gingival embrasure model in guinea pig](image)

3. Result and Discussion

3.1. Results

Confirmation tests on functional groups that were conducted using Fourier Transform Infra-Red (FTIR) in chicken ESM samples demonstrated that HA was successfully identified from the ESM samples. It was indicated by adsorption peak wave number functional groups Hydroxyl (–OH band at 3419 cm⁻¹), Carboxyl (–COOH band at 1634 cm⁻¹), and Ether (C–O–C saccharide unit at 1049 cm⁻¹) (Figure 2). Moreover, HA was also detected from ESM under UV light at 254 nm using TLC analysis.

Regarding the in vivo results, all the experimental procedures were well tolerated and did not affect the animals’ body weight. SPD gradually increased in both groups at day 1. Furthermore, SPD was observed increased at day 2 post-injection in groups injected with ESM extract. Meanwhile, as tooth eruption, the level of LPD was increased following tooth eruption in both groups. Interestingly, the AIP levels of the treatment group were found to be significantly higher than that of the control groups (P < .05) (Figure 3). These results suggested that ESM injection was significantly increased the growth of interdental papilla following orthodontic tooth movement.
Figure 2. FT-IR spectra of the specimens tested. There are –OH, –COOH, and C–O–C peak wave number was confirmed on the chicken eggshell membranes.

Figure 3. Mean (standard deviation) of AIP levels (mm) from 2 groups tested on day 0, 1, and 2 post-injection. *P<0.05 indicated a significant difference.

3.2. Discussion
Orthodontic tooth movement is a factor that may cause open gingival embrasures. To overcome these limitations, this study was conducted to develop a new biomaterial for tissue reconstruction and regeneration therapy in the case of open gingival embrasures. ESM is 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 [13]. A recent study revealed that HA is a major component of GAG in raw ESM, and the compositional analysis of raw ESM using high-performance liquid chromatography showed that 81% of GAG types contain HA [14]. HA has been widely used for both medical and dental therapy and has shown no evidence of contraindication or any interaction [8]. ESM contains the highest level of HA...
compared with other sources (synovial liquid, chicken comb, etc.), with the composition reaching 0.5%–10% of the total weight [9]. HA has evolved as the chosen material for tissue regeneration and reconstruction cases due to its ability to improve regeneration, retain the volume and integrity, and maintain tissue stability and elasticity [15]. The hydrophilic properties of HA allow its molecules to form hydrogen bonds with the carboxyl group surrounding it, thereby retaining water and hydrating the tissue and the weakened cell bonding against the matrix causing cell division and migration. HA is attached to the surface receptor CD44, a type of heparin proteoglycan containing sulfate that promotes cell adhesion or cell–matrix adhesion [16-17].

The result showed that HA from ESM significantly (\(P < 0.01\)) increased the AIP levels on day 1, and 2 post-injection compared to control groups. This phenomenon is in accordance with previous in vivo studies proving that HA potential to accelerates the healing process of alveolar bone after tooth extraction in vivo through stimulating the expression of osteogenic proteins [18]. Hyaluronic acid plays an important role in post-inflammatory tissue regeneration, facilitating cell migration and differentiation during tissue formation and repair. Hyaluronic acid may act as a scaffold for other molecules such as bone morphogenic protein-2 and platelet-derived growth factor, used in guided bone regeneration techniques and tissue engineering research [19].

A previous study conducted by Pi et al. using commercial HA filler injection showed an approximately similar result, proving that HA molecules have beneficial uses in tissue reconstruction and regeneration [11]. It is a limitation of our investigation that we have not used commercial HA as a control. Though the present study results are not as good as those of the previous study using commercial HA (since we cannot compare HA commercial and HA from ESM), the result of this study may suggest that HA isolated from ESM can be a good choice for an alternative material to treat the cases of tissue reconstruction and regeneration, including BT.

4. Conclusion
Based on the results of this study, we can conclude that chicken eggshell membrane containing hyaluronic acid has the potential effect to regenerate the interdental papilla construction after orthodontic tooth movement. However, further studies are required on an in vivo (studies on histopathology) and clinical level to confirm the efficacy and potency of chicken eggshell membrane in regenerating the open gingival embrasures in patients following orthodontic treatment.

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