Hyaluronic acid *vs.* physiological saline for enlarging deficient gingival papillae: a randomized controlled clinical trial and an *in vitro* study

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Background: loss of the interdental papillae leads to the formation of a black triangle, which compromises smile esthetics and contributes to food impaction and plaque accumulation. The aim of this study was to evaluate the efficacy of the injection of hyaluronic acid (HA) and compare it to that of physiological saline solution in the restoration of deficient gingival papillae *in vivo* and *in vitro*.

Methods: Twenty-four patients with 68 deficient gingival papillae were recruited for this clinical trial with a split-mouth design. The deficient gingival papillae on one side of the anterior maxilla were injected with HA, and those on the other side were injected with physiological saline solution. The heights of the gingival papillae and the areas of the black triangles were measured from clinical photographs obtained before and 6 and 12 months after treatment. Additionally, the proliferation and migration of gingival fibroblasts were evaluated after HA and physiological saline treatment by an *in vitro* study.

Results: the results revealed that the injection of HA yielded 0.198 and 0.28 mm gingival papilla increasement at 6 and 12 months, respectively, relative to the baseline (P<0.05). However, deficient gingival papillae also grew by 0.278 mm at 12 months in the group that received physiological saline solution (P<0.05). The injection of HA significantly improved deficient gingival papillae 6 months earlier than the injection of physiological saline solution. HA also significantly accelerated the proliferation and migration of gingival fibroblasts *in vitro*.

Conclusions: The present study confirms that the injection of HA could increase the height of gingival papillae for improving gingival papilla defects. However, the effect is not superior to that of physiological saline solution. This trial was registered in the Chinese Clinical Trial Registry (ChiCTR-ONC-17011781) (28/06/2017). http://www.chictr.org.cn/showproj.aspx?proj=19931

Keywords: Hyaluronic acid (HA); gingival papilla; gingival black triangle; physiological saline solution; gingival fibroblast

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Introduction

Interdental papillae, along with the optimal gingival contours, are crucial for ensuring smile esthetics. Gingival papilla defects in the anterior maxillary region lead to the formation of open gingival embrasures and negative spaces known as "black triangles", which are mostly undesirable to patients with high smile lines. There are several techniques, including restorative procedures (1), orthodontic treatments (2), interdisciplinary approaches (3) and a variety of surgical augmentation procedures (4-8), that can be used to reconstruct the missing gingival papillae. The first three options are costly and time consuming, while surgical techniques for augmenting the hard and soft tissues at the sites of the black triangles are invasive and yield unpredictable outcomes.

Recently, the injection of HA showed promising clinical efficacy in the treatment of gingival papilla defects in several studies. Becker et al. reported that small papillary deficiencies could be improved by injecting hyaluronic gel and that the enhancements were sustained for 6 to 25 months (9). Pi et al. also demonstrated in a rat model that the local injection of HA filler was a meaningful minimally invasive procedure for reducing the areas of black triangles (10). Awartani et al. demonstrated that interdental papilla loss could be improved after 6 months by the injection of HA gel in 10 patients with 17 black triangle sites (11). A study by Bertl et al., however, found that injection of HA adjacent to anterior maxillary implant-supported crowns did not result in any clinically obvious volume augmentation in deficient papillae (12). These conflicting findings indicate that validation of the efficacy of HA injection for the treatment of gingival papillary defects still requires higher-level clinical evidence. Meanwhile, basic research on the effects of HA on gingival connective tissue is necessary to unveil the underlying mechanisms.

HA is a non-sulfated glycosaminoglycan that is present in many body fluids. It maintains extracellular matrix elasticity and tissue hydration. Asparuhova et al. illustrated that hyaluronan (Regedent AG, Zurich, Switzerland) enhances the proliferation and migration of gingival fibroblasts, which are the main cell type in gingival connective tissue (13). The products of HA also increase the expression of genes encoding type III collagen and transforming growth factor-β3 (13), which partly explains the underlying mechanism by which HA in resolves gingival papillary defects. Unfortunately, no clinical study has been conducted on these products to confirm their clinical effects.

In our previous study (14), we verified the remarkable effectiveness of hyaluronic acid (HA) gel (Qi Sheng Biological Agent Company Limited, Shanghai, China) injections in restoring the deficient gingival papillae of natural teeth, especially in patients with thick gingival biotypes. Nevertheless, that study had several shortcomings, such as a small sample size and a lack of a control group. In this study, we further evaluated the efficacy of HA gel injections in the improvement of deficient gingival papillae and the reduction of black triangles and compare it to that of the injection of physiological saline solution with a randomized controlled split-mouth design. We also examined the proliferation and migration of gingival fibroblasts after stimulation with the same HA product and physiological saline product in vitro. We present the following article in accordance with the CONSORT reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7599).

Methods

Study population and inclusion criteria

Our study was designed based on the guidelines from the Consolidated Standards of Reporting Trials (CONSORT) statement (http://www.consort-statement.org/) and was registered in the Chinese Clinical Trial Registry (ChiCTR-ONC-17011781) http://www.chictr.org.cn/showproj.aspx?proj=19931. We confirmed that all methods were performed in accordance with the relevant guidelines and regulations. In this trial, 24 patients with 68 gingival papilla defects were recruited.

The inclusion criteria were as follows: (I) adults (20–70 years old); (II) no systemic disease such as hypertension, coronary artery disease, stroke, or Type II diabetes that would affect periodontal treatment; (III) no fixed prostheses or caries on the studied teeth; (IV) good oral hygiene (full-mouth plaque score <20%) (15); (V) two or four symmetrical gingival papilla defects (Class I, in which the tip of the interdental papilla lies between the interdental contact point and the most coronal extent of the interproximal cemento-enamel junction (CEJ), or Class II, in which the tip of the interdental papilla lies at or apical to the interproximal CEJ but coronal to the apical extent of the facial CEJ (16) in the anterior maxilla; (VI) healthy periodontal tissue or well-controlled inflammation, which is defined as <10% bleeding sites with probing depths ≤3 mm (17); (VII) no history of periodontal surgery in the last 6 months; (VIII) no smoking;
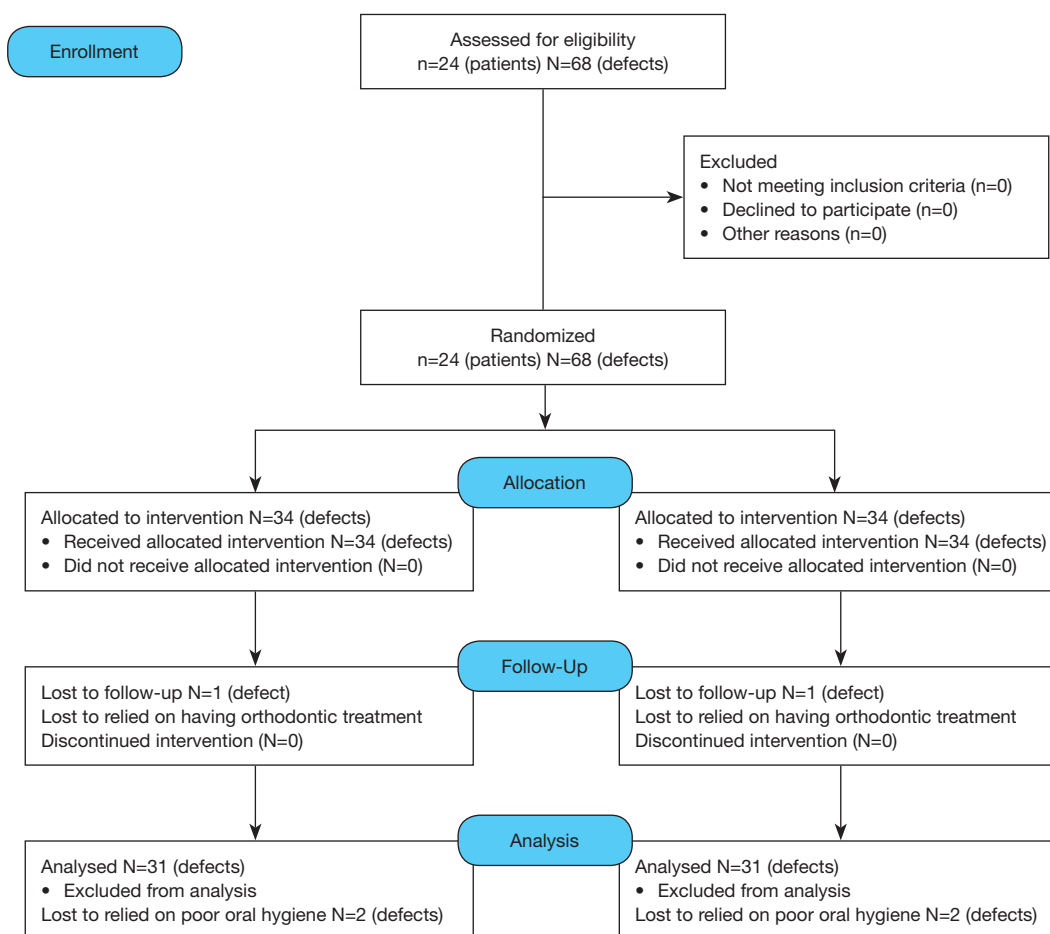
Figure 1 Flow chart of the clinical trial.

(IX) no history of regular medication intake such as calcium channel blockers or cyclosporin A that could affect gingiva metabolism (18).

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Shanghai Ninth People's Hospital Human Studies Ethics Committee (No. 2016-229-T178). All participants were informed of the objective and risks of the study. Candidates were required to provide signed consent to participate.

Study design

The study used a split-mouth design, as shown in *Figure 1*. All candidates received initial periodontal therapy, which involved the use of scaling, root debridement and the provision of oral hygiene instructions. After one month, the candidates were reevaluated, and those who fulfilled the inclusion criteria were recruited into the study. Blinding was implemented for the trial participants, the image assessors, who made the measurements on the images, and the data collections. An independent researcher created random allocation cards with Microsoft Excel. The random assignment of an odd number indicated that the left side would be used for the test group (HA group) and the right side for the control group (physiological saline solution group). The random assignment of an even number indicated that the right side would be used for the test group (HA group) and the left side for the control group (physiological saline solution group). Yifan Wu performed the follow-up examinations 6 and 12 months after the injections.

Standardized photography and measurement

Prior to the injections, a standardized clinical photograph of

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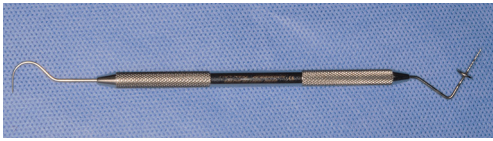
Figure 2 The intraoral scale calibration custom probe with a stop.
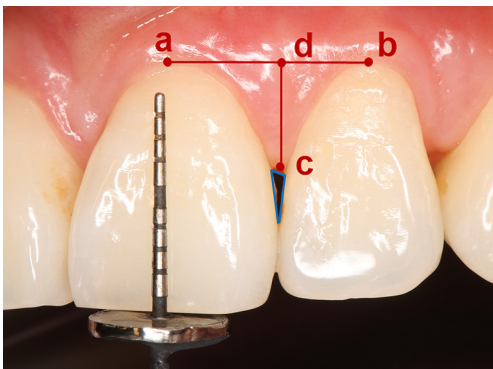
Figure 3 Description of the methodology used to measure the papilla height and papilla triangle areas. Line ab represents the line connecting the highest point of the adjacent teeth's crowns with the gingival papilla defect. Point c represents the top of the deficient gingival papilla. Line cd is vertical to line ab. cd represents the papilla height, and the papilla triangle area is marked with a blue line.

the gingival papilla defects was taken with a Canon EOS5D Mark III digital camera. The lens of the camera was a Canon EF 100m f/2.8L IS USM, and the camera parameters were ISO 200, f/32 and 1/160s.A 1:1 magnification ratio and 25 cm fixed distance were maintained during the photo shoot. Meanwhile, one assistant checked that the camera was perpendicular to the long axis of the adjacent teeth and parallel to both the participant's Frankfurt horizontal plane and the ground. An intraoral scale calibration custom probe with a stop was used in the study (*Figure 2*). The stop could ensure the probe is parallel to the long axis of the tooth.

Afterwards, the height of each gingival papilla (primary outcome measure) and the area of each black triangle (secondary outcome measure) was measured on the clinical image with Digimizer version 4.2 software (Med Calc Software, Belgium), as described in previous study (14). A summary description of the methodology used to measure the papilla height and triangle areas is illustrated in *Figure 3*. Training for measurement from the clinical images was performed by measuring the papilla height and papilla triangle areas on 10 clinical images that were not associated

with the clinical trial.

Injection procedure

All injection procedures were performed under anesthesia by the same experienced periodontist. The control sites, located on one side of the mouth, were injected with physiological saline solution at the bases of the deficient papillae. The test sites, located on the other side of the mouth, were injected with 16 mg/mL HA gel (Qi sheng Biological Agent Company Limited, Shanghai, China) at the bases of the deficient papillae. Each site received 0.05–0.1 mL of solution. The injection procedures were repeated 3 and 6 weeks after the initial injections.

In vitro study

Cell culture and identification

Human gingival (HGF) fibroblasts were obtained from 3 systemically and periodontally healthy patients (<10% bleeding sites and probing depths ≤3 mm) who had undergone crown lengthening. In advance of the procedure, the patients signed informed consent forms. The *in vitro* study was approved by Shanghai Ninth People's Hospital Human Studies Ethics Committee. The gingival tissue obtained was minced into 1 mm pieces and cultivated in a 21 cm^2 culture dish in DMEM, Dulbecco's modified Eagle's medium (HyClone, USA) supplemented with 10% fetal calf serum (GIBCO, USA). Cells between passages 4–7 were used in the study. Gingival fibroblasts were identified with morphological observation and immunochemistry.

Cell proliferation assay

The proliferation rate of HGF cells was determined by CCK-8 (Dojindo, Shanghai, China). HGF cells were inoculated in 96 well plates at a density of $2 \times 10^3/100$ μL per well. After 24 h of incubation, 0% HA, 1% HA, 3% HA and 6% HA were added to the 96-well plates. The cells were incubated for 1 day, 2 days, 3 days and 4 days, respectively. After CCK-8 was added to the wells, the plate was placed into an incubator for 2 h, and the absorbance value was measured at 450 nm with a Microplate Reader. Three independent experiments were performed with the cells obtained from the three different patients.

Cell migration assay

After serum-free starvation for 24 h, the cells were resuspended in DMEM containing 1% FBS, and the cell

concentration was adjusted to 1×10^5 cells/mL. A 150 μL cell suspension was inoculated in the upper chamber (Corning, New York, USA). A total of 700 μL of 0%, 1% and 13% HA, which were all diluted in DMEM with 1% FBS, was added to the lower chamber. After 12 h of cultivation, the non-migrated cells on the upper chamber surface were wiped with cotton swabs. After 30 minutes of methanol fixation, 0.1% crystal violet staining was performed. The mean values of 3 visual fields were calculated. Three independent experiments were performed with the cells obtained from the three different patients.

Data analysis

Data analysis was performed with SPSS (Version 23.0, IBM, Armonk, NY, USA). Differences were considered significant when their P values were less than 0.05. Descriptive statistics were calculated and are presented as the mean ± SD. For the clinical study, the gingival papilla heights and the black triangle areas were compared with repeated-measures analysis of variance (ANOVA). In detail, first, the studentized residuals were generated and analyzed. The results indicated that the data from each group had no outliers (no value beyond ±3) and followed a normal distribution (all P>0.05 by the Shapiro-Wilk test). If $P_{Mauchly's\ test\ of\ sphericity} \geq 0.05$, the P values of sphericity assumed were adopted; if $P_{Mauchly's\ test\ of\ sphericity} < 0.05$, and epsilon (ε) <0.75, the P values of Greenhouse-Geisser were adopted; if $P_{Mauchly's\ test\ of\ sphericity} < 0.05$ and epsilon (ε) >0.75, the P values of Huynh-Feldt were adopted. Bonferroni correction was applied to the multiple comparisons. One-way analysis of variance (ANOVA) was adopted in the *in vitro* study. P values <0.05 were considered significant.

Results

Twenty-four patients with a total of 68 gingival papilla defects were included in the study. Thirty-four gingival papilla defects were in the test group (N=34) and treated with HA gel, and 34 gingival papilla defects were in the control group (N=34) and treated with physiological saline solution. One patient discontinued participation in orthodontic treatment. Two patients' data were removed from the analysis because of poor oral hygiene, which led to gingival swelling. The study ended with completion of the 12-month follow-up and the statistical analysis of the data of the remaining 21 patients with 62 gingival papilla defects. The patients' ages ranged from 28 to 63 years (average

41.3±7.73 years), and 2/21 were males (*Table 1*).

As shown in *Table 2*, the average increases in gingival papilla height were 0.198±0.34 and 0.280±0.38 mm at 6 and 12 months respectively, in the test group. In the control group, the average increases in gingival papilla height were 0.135±0.39 and 0.278±0.45 mm at 6 and 12 months respectively. The average decreases in the black triangle area were 0.26±0.42 and 0.45±0.54 mm² at 6 and 12 months respectively, in the test group. The average decreases in black triangle area were 0.15±0.37 at 6 months and 0.32±0.50 mm² at 12 months in the control group. The intragroup comparisons for the test group indicated statistically higher gingival papilla heights and statistically smaller black triangle areas between 6 months and baseline (*Table 2*, P=0.011 for the gingival papilla height, P=0.007 for the black triangle area). Similar results were shown between 12 months and baseline (*Table 2*, P=0.001 for the gingival papilla height, P<0.001 for the black triangle area). The intragroup comparisons for the control group indicated no significant difference in either the gingival papilla height or the black triangle area between 6 months and baseline (*Table 2*, P=0.199 for the gingival papilla height, P=0.098 for the black triangle area). However, significantly different results were observed between baseline and 12 months (*Table 2*, P=0.006 for the gingival papilla height, P=0.004 for the black triangle area). Intergroup analysis revealed that there was no significant main effect for the changes in gingival papilla heights or the area of black triangles between the test group and the control group (*Table 2*, P=0.078 for gingival papilla height, P=0.826 for black triangle area). Clinical photographs of representative patients are shown in *Figure 4*.

HGFs were cultivated with the explant culture method, and fibroblast-like cells could be observed around the tissue edge after 3 days. The cells were passaged after reaching 80–90% confluence. The obtained cells were spindle shaped under a microscope (*Figure 5*). The immunochemistry results indicated that the cells were positively stained with vimentin antibody and negatively stained with cytokeratin antibody (*Figure 5*). The cultured cells were confirmed to be derived from the mesoderm and were not mixed with epithelial cells.

The CCK-8 results showed that gingival fibroblasts proliferated significantly after stimulation with 1% HA, 3% HA and 6% HA for 3 days and 4 days relative to the control group (*Figure 6*). Cell migration assays confirmed that gingival fibroblasts migrated significantly in the 13% HA group relative to the 1% FBS control group and 1% HA

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Table 1 Characteristics of the study population

Patient	Age	Gender	Site		Class	
			Control group	Test group	Control group	Test group
1	32	Female	1	4	Class I	Class I
2	43	Male	4	1	Class I	Class I
3	63	Female	4	1	Class II	Class II
4	32	Female	3,4	1,2	Class II	1= Class I, 2= Class II
5	28	Female	2	3	Class II	Class II
6	36	Male	3,4	1,2	Class I	Class I
7	47	Female	3,4	1,2	Class II	Class II
8	41	Female	1,2	3,4	Class I	Class I
9	45	Female	3,4	1,2	Class II	Class II
10	42	Female	2	3	Class II	Class II
11	45	Female	3	2	Class II	Class II
12	45	Female	1,2	3,4	Class II	Class II
13	38	Female	3	2	Class II	Class II
14	48	Female	2	3	Class II	Class II
15	36	Female	1	4	Class II	Class II
16	34	Female	3,4	1,2	Class I	Class I
17	39	Female	1,2	3,4	Class I	Class I
18	52	Female	2	3	Class I	Class I
19	38	Female	1,2	3,4	Class I	Class I
20	43	Female	3,4	1,2	Class II	Class II
21	41	Female	3	2	Class II	Class II

Site 1= interdental papilla between maxillary right canine and maxillary right lateral incisor; Site 2 = interdental papilla between maxillary right lateral incisor and maxillary right central incisor; Site 3 = interdental papilla between maxillary left lateral incisor and maxillary left central incisor; Site 4 = interdental papilla between maxillary left lateral incisor and maxillary left canine.

Table 2 Clinical data for gingival papilla treated in control and test group at baseline and after 6 and 12 months

| Parameter/treatment	Baseline	6 months	Δ6 months-BL	P value[a]	12 months	Δ12 months-BL	P value[b]	P value[c]
The height of gingival papilla (mm)								0.078
Control group (N=31)	2.99±1.42	3.12±1.36	0.135±0.39	0.199	3.26±1.39	0.278±0.45	0.006*	
Test group (N=31)	3.25±1.30	3.45±1.27	0.198±0.34	0.011*	3.53±1.25	0.280±0.38	0.001*	
The area of black triangle (mm^2)								0.826
Control group (N=31)	1.78±1.31	1.63±1.26	−0.150±0.37	0.098	1.46±1.26	−0.320±0.50	0.004*	
Test group (N=31)	1.90±1.37	1.65±1.32	−0.260±0.42	0.007*	1.45±1.16	−0.450±0.54	<0.001*	

Data presented as mean ± standard deviation. *, indicates P<0.05. P value[a]: 6 months versus baseline within group using one-way repeated ANOVA corrected by Bonferroni; P value[b]: 12 months versus baseline within group using one-way repeated ANOVA corrected by Bonferroni; P value[c]: test group versus control group using two-way repeated ANOVA.
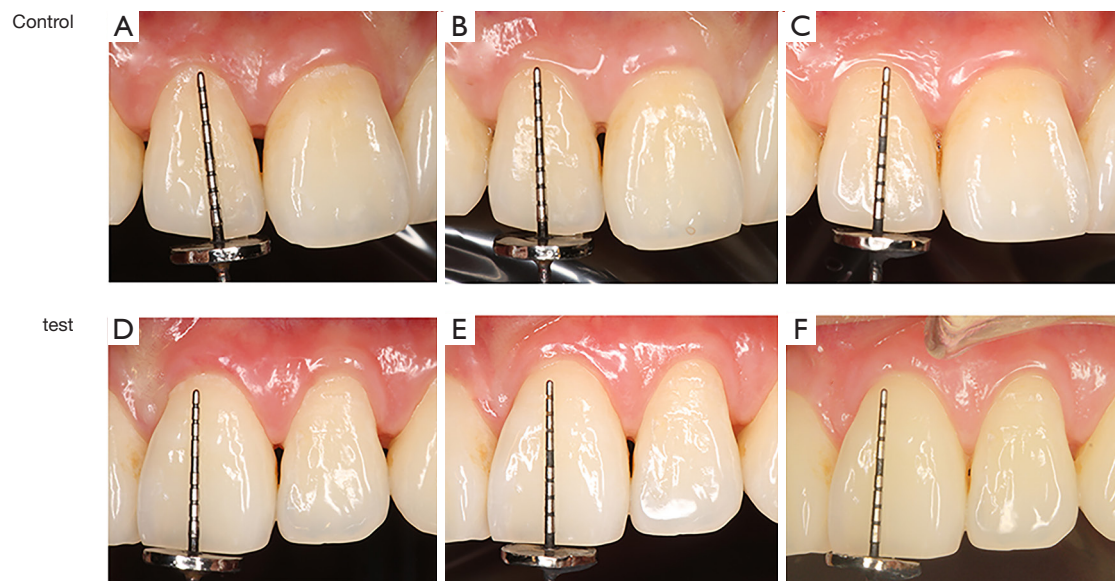
Figure 4 Clinical photographs of representative patients treated with injections of physiological saline solution (control group) and hyaluronic acid gel (test group). A deficient gingival papilla between the right maxillary incisor and the right maxillary lateral incisor is shown after treatment with a physiological saline solution injection at baseline (A) and after 6 months (B) and 12 months (C). A deficient gingival papilla between the left maxillary incisor and the left maxillary lateral incisor is shown after treatment with a hyaluronic acid gel injection at baseline (D) and after 6 months (E) and 12 months (F).
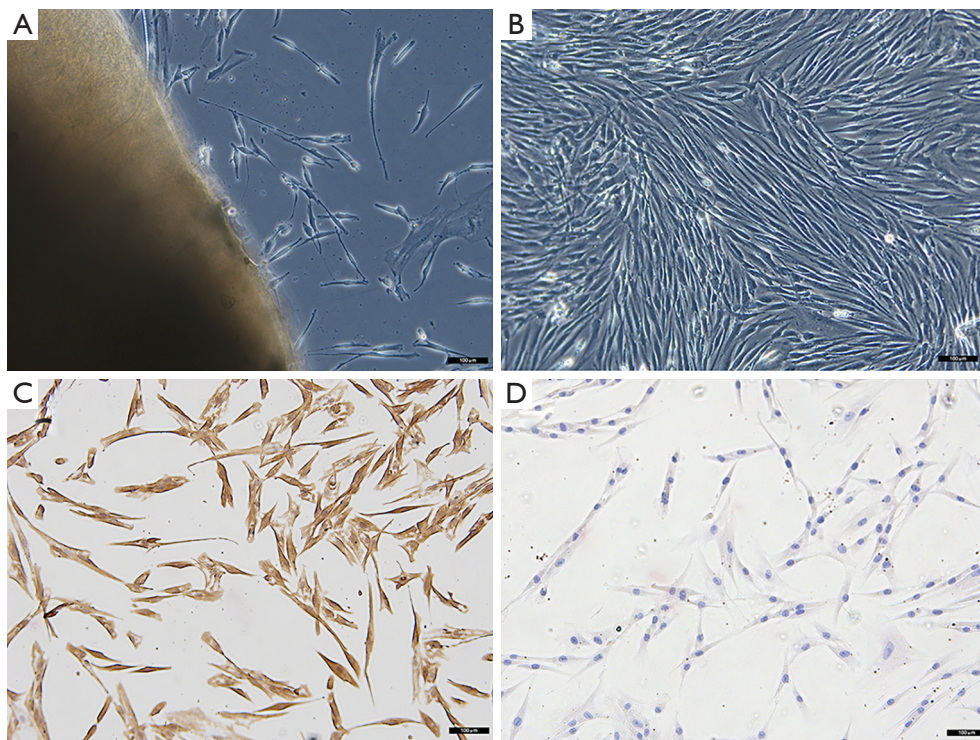
Figure 5 hGFCs culture and identification. (A) Primary hGFCs cultured *in vitro*, scale bar 100 μm. (B) 2nd passage of the hGFCs, scale bar 100 μm. (C) hGFCs positive for vimentin antibody, scale bar 100 μm. (D) hGFCs negative for cytokeratin antibody, scale bar 100 μm.

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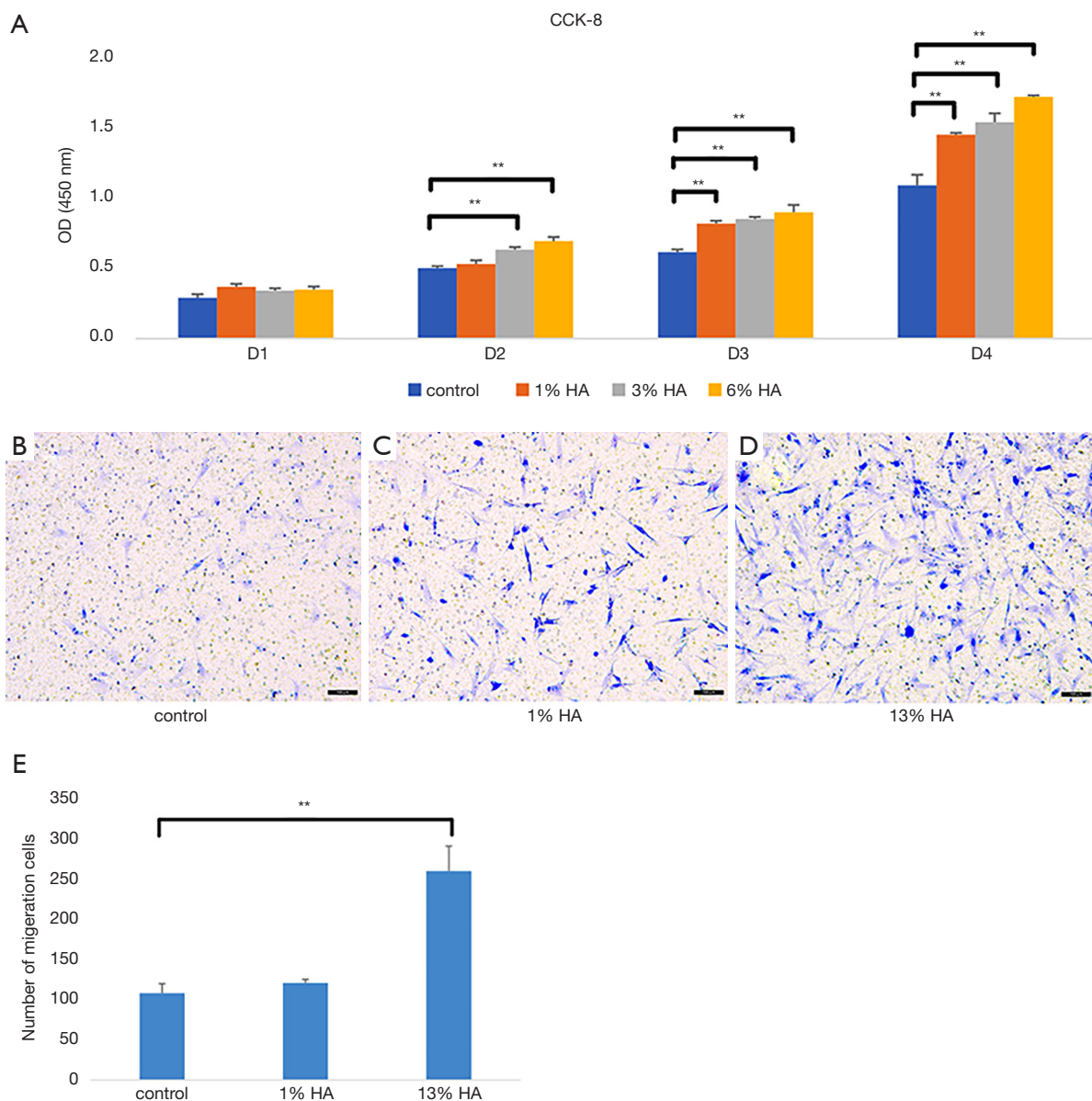
Figure 6 The proliferation and migration of hGFCs after HA treatment. (A) hGFCs were stimulated with 0%, 1%, 3% and 6% HA for 1, 2, 3 and 4 days. Asterisks indicate significant differences between the control group and HA stimulated group at different time points, **P<0.001. (B,C,D) Migration of hGFCs toward 0%, 1% and 13% HA, respectively, was evaluated with Transwell migration assay. The cells were stained with 0.1% crystal violet. Representative images are shown, scale bar 100 μm. (E) Cell migration was qualified by counting the cells on the lower side of the filter in three visual fields. Values are the mean ± SD (n=3), **P<0.001. HA, hyaluronic acid.

group (*Figure 6*).

Discussion

HA, also called hyaluronan, is a major natural carbohydrate component of the extracellular matrix and is distributed widely in the joints, skin, eyes and connective tissues. The products currently in use and on the market are usually extracted from animal tissues such as rooster combs or obtained from certain bacterial strains (19). Meanwhile, the extracted HA must be chemically modified, such as via a cross-linking process, which could prevent quick

degradation and prolong the effective time. The HA used in our study is produced from microbial fermentation.

Recently, many studies have reported that local application of HA adds to the benefits of nonsurgical and surgical periodontal therapy because of its anti-inflammatory, antiedematous and antibacterial effects (20). HA is also labeled a dermal filler by plastic surgeons because of its unique ability to bind and retain water molecules (21). Hence, it has also been introduced to the field of esthetic dentistry to augment the gingival papilla and improve the esthetic aspects of the black triangles between teeth (9). Nevertheless, most of the studies on this topic have been case series or have lacked a control group, and only two RCTs with a parallel group design have been conducted. The results of these two studies are contradictory. Abdelraouf *et al.* demonstrated a significantly greater mean increase from baseline papilla height and decrease from baseline black triangle area in the HA group than in the control group after 3 and 6 months (22). However, the author did not clearly define tooth morphology or gingival biotype in the inclusion criteria, which are two important factors affecting the effects of gingival papilla treatment. Bertl *et al.* revealed that there was no clinically conspicuous increase in the volume of deficient papillae alongside implant-supported crowns in the anterior maxilla 6 months after the injection of HA (12). The different inclusion criteria, injection time points and HA products may account for the heterogeneity between the two studies.

In our previous study (14), we verified the remarkable effectiveness of HA gel (Qi Sheng Biological Agent Company Limited, Shanghai, China) injections in restoring the deficient gingival papillae of natural teeth, especially in patients with thick gingival biotypes. Nevertheless, that study had several shortcomings, such as a small sample size and a lack of a control group. Our present study sought to further evaluate the clinical results of HA gel injections versus saline injections in the treatment of gingival papilla defects with parallel *in vivo* and *in vitro* experiments. This is the first study with a split-mouth design in the literature on this topic; this design could reduce the impact of interindividual variability in patient characteristics that may impact the effects of treatment, such as age (23), crestal alveolar bone height (24), contact point (25), tooth shape (24), and interradicular distance (26). The results indicated that the HA gel group had improved significantly at 6 and 12 months relative to the baseline. This is also the first study to demonstrate the clinical efficacy of HA from both clinical and basic research perspectives. The

two results support each other and illustrate that HA can effectively accelerate the proliferation and migration of gingival fibroblasts, ultimately increasing the height of deficient gingival papillae.

The improvement in deficient gingival papillae 12 months after saline injection is surprising. The underlying mechanism may be related to the natural creeping of gingival papilla and soft tissue regeneration caused by local tissue pressure. Previously, some studies reported that the application of compressive or retractive force could induce enlargement of the interdental papillae, especially at the site of closure of the extraction space. Nan *et al.* also demonstrated that mechanical force could promote the proliferation and extracellular matrix synthesis of human gingival fibroblasts (27). We assume that the injection of HA or saline solution applies some compressive force to gingival fibroblasts, which may also accelerate the proliferation of the cells and enhance the formation of ECM, improving gingival papilla defects.

This study has some limitations, such as the lack of information regarding patient satisfaction with and perception of the procedure, the lack of three-dimensional measurements of the gingival papillae, which would have improved the precision with which we could monitor the changes in black triangle size, and the overwhelming predominance of female patients, which prevented us from considering the influence of sex hormone levels on gingival papilla height and the inclusion of only maxillary sites. Additionally, the sample size is the most crucial issue in clinic trials, and we lacked a specific priori calculation process for sample size determination in the present study. However, we conducted back testing to evaluate the statistical power based on the data from this study. For the intragroup comparison, the statistical power obtained based on repeated measures was 98%, indicating that there was a 98% chance of correctly rejecting the null hypothesis of no difference between the follow-up time points and baseline with a total of 60 gingival papilla defects. However, for the overall comparison between the HA gel intervention and the physiological saline solution control, the statistical power the statistical power obtained based on repeated measures was 64%, indicating that there was a 36% chance of falsely retaining the null hypothesis of no difference with a total of 60 gingival papilla defects. Therefore, the result that there is no statistically significant additional benefit in the improvement of gingival papilla defects after HA injection compared with physiological saline injection must be interpreted with caution, and a larger sample size study is needed in the future.

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Conclusions

The present study confirms that the injection of HA could increase the gingival papilla height for gingival papilla defects and reduce the area of the resulting black triangles. However, there is no statistically significant additional benefit in the improvement of gingival papilla defects after HA injection comparing with physiological saline injection. In vitro study, the same HA product significantly accelerated the proliferation and migration of gingival fibroblasts. In the future, a long-term multicenter clinical study should be carried out to standardize the protocol for HA injection, optimize the injection concentration and interval, and identify the determinants affecting the clinical outcomes.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the CONSORT reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-7599

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-7599

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Shanghai Ninth People's Hospital Human Studies Ethics Committee (No. 2016-229-T178). All participants were informed of the objective and risks of the study. Candidates were required to provide signed consent to participate.

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