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Effect of Hyaluronic acid and Chitosan hydrogel in management of Black triangle (clinical and experimental study)

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Abstract

Hyaluronic acid (HA) has been widely used to overcome esthetic problems associated with open gingival embrasures or black triangle with the drawback of rapid degradability. Chitosan is an example of natural biodegradable polymer composite that has been used as a scaffold for HA. The aim of the present study was to evaluate a hybrid hydrogel of HA and Chitosan in the management of open gingival embrasure clinically and histologically. A total of 36 patients with 42 open gingival embrasures were divided randomly into two equal groups. Group A was injected with 0.2 ml of hyaluronic acid gel. Group B was treated with 0.2 ml of a mix of (50% hyaluronic acid+ 50% chitosan hydrogel). Clinical parameters included plaque index (PI), bleeding index (BI), probing depth (PD), height and surface area of the black triangle. Parameters were evaluated at baseline, after 6 and 9 months following the second injection. For histological evaluation, 25 adult rats were used in this study in three groups I, II and III. Control group (n=5) was injected with Phosphate buffered saline (PBS). Group II (n=10) was injected with 0.1 ml of HA. Group III (n=10) received an injection of 0.2 ml of a mix of (50% hyaluronic acid+ 50% chitosan hydrogel). Histological changes of the gingival tissue were examined using H&E and Masson's trichrome after 6 and 9 post-injections. Regarding clinical parameters, the height and surface area of the black triangle in each group showed significant decrease from base line to 6 months interval in both groups. Histological evaluation revealed an increase in the thickness of the gingival tissue in both groups II and IIII with an increase in the amount of collagen and elastic fibers although it was more evident in group III. Both hydrogels are safe and biocompatible to be used in management of black triangle. Though, hybrid hydrogel of HA and chitosan has a more long-lasting effect.

Keywords: Hyaluronic acid, Chitosan hydrogel, Black triangle.

Full length article *Corresponding Author, e-mail: Ahmedabdallahuss@yahoo.com

1. Introduction

Esthetic achievement is a primary goal of both the patient and the dentist. One of the main esthetic problems is black triangle or open gingival embrasure. It is an empty space below the interproximal contact that is not filled with gingival tissue [1]. It is a common occurrence affecting more than one third of adults [2]. Open gingival embrasure compromises esthetics and promotes periodontal diseases due to food impaction [3]. Age, periodontal disease, periodontal surgery, and orthognathic treatment may contribute to the development of black triangle [2]. Several surgical techniques have been proposed to manage loss of gingival embrasure. Surgical management of black triangle has been elusive due to limited blood supply of the interdental tissue. Moreover, the results are unpredictable [4]. Therefore, great attention has been directed towards using minimal invasive techniques for interdental gingival tissue reconstruction as injection of fillers and biological preparations [5]. Hyaluronic acid is a polysaccharide present in body tissues such as skin, hyaline cartilage, synovial fluid, and the eye vitreous humor acting as a shock absorbing fluid [6]. It is a non-sulfated glycosaminoglycan of high molecular weight composed of repeating disaccharide units of N-acetyl-D glucosamine and D-glucuronic acid. It has a physiological role in preserving the extracellular matrix structure of connective tissue, synovial fluid, and other tissues. Moreover, It regulates intercellular activities as cell-cell adhesion and cell signaling [7]. Within the oral cavity, HA can be found in the gingiva and periodontal ligament. Cementum and alveolar bone contain small amounts of HA [8]. The biomedical applications of HA include neurosurgery, cutaneous wound healing,

cartilage tissue engineering, cancer treatment, ophthalmology, and cosmetics because of the virtue of its angiogenic, immunomodulatory, anti-coagulant and targeted transport activities [9]. In dentistry, HA has been used in regenerating soft and hard tissues, treating oral ulcers, sinus lifting and management of inflammatory conditions of gingiva and periodontal tissues [10]. Unfortunately, HA is rapidly degradable and has low mechanical strength. Consequently, this adversely affects its effectiveness. To improve its half-life and extend its application, modifications of HA have been encountered [11]. Chitosan is a natural polysaccharide composed of glucosamine and N-acetyl-glucosamine [12]. It is obtained by deacetylation of chitin, a natural polysaccharide found extensively in the cell wall of some bacteria and fungi as well as the exoskeletons of arthropods such as crustaceans. Due to its properties of biocompatibility, biodegradability, non-toxicity, and hemostatic action, it has various applications in tissue engineering, wound healing and drug delivery [13]. In tissue engineering, scaffolds of biological degradable material having the advantages of both excellent malleability and filling capability have been used to promote tissue growth. A chitosan-based hydrogel or HA has gained much attention in the recent years as an injectable biomaterial scaffold for tissue regeneration in dentistry [14]. The present prospective clinical trial aimed to evaluate the effect of injecting hybrid hydrogel of HA and chitosan to manage black triangles clinically and histologically.

2. Patients and methods

This study comprised both clinical and histological evaluation of the application of a chitosan-based hydrogel of HA. The study protocol was approved by the Ethics Committee, Faculty of Dentistry, Minia University. Committee No. (94), decision No. (738).

2.1. Clinical study

2.1.1. Patient selection

The current study was conducted on a total of 36 patients with 42 deficient papillae recruited from the outpatient clinic of the oral medicine, Oral diagnosis, and Periodontology Department, Faculty of Dentistry, Minia University. All participants were provided with detailed information of the clinical trial and a signed consent form was obtained from each participant. Every patient has at least one deficient papilla in the anterior area. Clinical examination revealed that the contacting teeth were in close approximation. Eligibility criteria were as follows: Adult (25-45 years), Systemically healthy, with at least one maxillary or mandibular interdental space exhibiting class I or II interdental papilla loss [15] in the anterior area with good plaque control and good oral hygiene. Exclusion criteria were as follows: history of allergic reaction to injectable filler, smoking, pregnant and lactating women, medication affecting gingiva or wound healing, alcoholics, patient under orthodontic treatment or had orthodontic treatment in last 6 months.

2.1.2. Patients grouping

The total 42 sites were divided randomly by using toss coin into two equal test groups (A and B) each having 21 sites for injection. For group A (HA group): Restylane-L® a commercially available hyaluronic acid gel was used to inject the deficient papilla. For group B (HA and chitosan group): A mix of 50% hyaluronic acid (Restylane-L) + 50% chitosan hydrogel was used to inject the deficient papilla.

2.2. Chitosan hydrogel preparation

2.2.1. Material

Chitosan (middle-viscous with degree of deacetylation ≈ 80), MW 250–400 kDa range were purchased from Fluka BioChemika. antrez® S-97 (GAN) (acid form of methylvinylether and maleic anhydride copolymer) (Mw = 1.2 × 106 Da), was provided by Ashland (Tadworth, Surrey, UK).

2.2.2. Preparation of chitosan hydrogel

Chitosan solution (1% w/v) was prepared by slow dissolving of 800 mg chitosan in 80 ml acetic acid aqueous solution (0.15 M) using mechanical stirring. The pH of the resultant chitosan solution was adjusted to 4.8. The prepared chitosan solution was left at 4^{o}C for two hours for degassing [16].

2.3. Treatment protocol

2.3.1. Pre-operative Phase

- Oral hygiene instructions were given to all participants and when necessary, supragingival debridement prior to study procedures was performed.
- Subsequently, Digital clinical photographs were taken of the deficient papillae. The patients were sitting in an upright position, looking straight ahead. The Frankfort plane of the patient was parallel to the ground.
- The photographs were captured perpendicular to the teeth adjacent to the deficient papilla. The surface area of the black triangle was assessed using standardized clinical photographs analyzed by an image analysis program (adobe photoshop cc 2019).
- The photographs were imported to photoshop. The contrast of the photographs was adjusted to ensure that the borders of the black triangle were distinct. This was achieved by turning the area of the black triangle into completely black while the rest of the image turned white. The actual size corresponding to a pixel is different on photographs taken at different times. To reduce errors based on magnification, an unc-15 color-coded graduated periodontal probe was used as a scale for calibration to calculate the pixels value in mm then the base and height of the black triangle were measured in mm **(figure 1 a&b).** The surface area of the black triangle (in mm^2) was calculated using the formula (0.5 x height x base)
- Clinical measurement of the height of the black triangle was done by measuring the distance between the deficient papilla tip (PT) and the most apical extent of the contact area (CP) using the periodontal probe to the nearest 0.5 mm. For standardization of measurements, a customized stent was fabricated for proper positioning of the periodontal probe at each time interval.

2.3.2. Drug application: (figure 1 c)

For group A (HA group): After administration of a local anesthesia, a 30-gauge disposable plastic insulin syringe was used to inject 0.2 mL of Restylane® a commercially available and FDA-approved hyaluronic acid gel 2-3 mm apical to the tip of the involved papillae and

directed coronally with an angulation of 45° to the long axis of the tooth and the bevel directed apically. Then, the papilla was lightly molded in an incisal direction for 1 minute using gauze. A second injection (3 weeks later) was applied using the same technique. For group B (HA and chitosan group): 0.2 ml (50% of hyaluronic acid + 50% of chitosan gel) was injected on the deficient papillae of group B using the same injection method. The second injection (3 weeks later) was applied using the same technique. The patients were requested not to brush their teeth on the day of injection and to resume oral hygiene measures on the following day by a soft-bristled toothbrush. The patients were asked not to use dental floss at the treatment sites for 4 - 6 weeks and to use 0.12% chlorhexidine mouthwash [17].

2.4. Clinical assessment

Follow up data including Probing depth, plaque index, gingival index, height of black triangle and the surface area of the black triangle at the sites of papillary deficiency were assessed at base line (before injection), 6 and 9 months after injection for all the respective areas. For assessment of black triangle area, the clinical photographs were taken under the same lighting conditions and camera setting. Strict care was taken to ensure that the same up-down and right-left shooting positions were reproduced at different time intervals (Fig 2).

2.4.1 Experimental animal protocol

25 adult male rats (200–250 g) were provided from animal house, Deraya University, Minya, Egypt. The animals were maintained under the following conditions: 25± 2 °C, 50–60% humidity and natural light–dark cycles with free access to food and water. All methods were performed in accordance with the relevant guidelines and regulations. The rats were randomly divided into the following three groups: control group (n = 5) injected with phosphate buffered saline (PBS), group I (n = 10) injected with HA (10 µL) and subdivided into group Ia (Sacrificed after 6 months) and group Ib (sacrificed after 9 months), and group II (n = 10) injected with HA and chitosan hydrogel (10 µL) and subdivided into group IIa (sacrificed after 6 months) and subgroup IIb (sacrifices after 9 months). The animals were anesthetized with an intraperitoneal injection of avertin (0.02 mL/g bodyweight) before injection which was done 1–2 mm below the interdental papillae crest with a 30 G needle (Fig 3).

2.4.2. Tissue Preparation

The control group, group Ia, and group IIa were sacrificed using CO_2 gas 6-month post-injection and subjected to histological analysis. The remaining rats were sacrificed on 9-month post- injection. The interdental papillae were dissected from the mice and immediately fixed in 4% paraformaldehyde (PFA) at 4 °C for 24 h. The specimens were dehydrated and embedded in paraffin. The paraffin-embedded samples were subjected to serial coronal sectioning for further histological analysis (Table 1).

2.4.2.1. Histological study

The sections, 4 µm thick, were processed for hematoxylin and eosin staining (H&E) for examination of the tissue structure using the standard technique [18]. Other sections were stained with Masson's trichrome for examination of collagen fibers [19].

2.4.2.2. Morphometric analysis

Photomicrographs of fields were obtained with a digital camera (Cybershot DSC-W300; Sony, Manaus, AM, Brazil) connected to an optical microscope (Leica, Germany) (magnification × 200) and analyzed using analysis software (Image J, 1.41a, NIH, USA). For Masson's trichrome stained sections, the area percentage of collagen fibers in the submucosal tissues (blue coloration) without considering intensity was measured in 5 microscopic field of each slide. The mean percentage of collagen fiber area was calculated from the five fields per subject. The histological examination was blind to group allocation (Table 2).

2.5. Statistical Analysis

One way ANOVA and Tukey's post hoc test were used for all comparisons of collagen fiber percentages among the three groups. Descriptive statistics were presented for the animals in mean ± SD. P-value of less than 0.05 was considered statistically significant. Mann Whitney test was used to detect the differences between groups.

3. Results

3.1. Clinical assessment

The study was conducted on a total of 36 patients with 42 deficient papillae that were randomly and equally allocated to both tested groups (i.e., 21 deficient papillae per group). Out of 17 patients in group I, there were 14 (82%) females and 3 (18%) males. While in group II, there were 13(68%) females and 6 (32%) males. The mean age of group I was (34.29±6.13) years, while in group II, it was (33.57±6.08) years. There was no significant difference between both groups regarding sex distribution (p=0.542) and age (p=0.860). Regarding the height and surface area of the black triangle in each group, results showed significant decrease from base line to 6 months interval in both groups. While at 9 months, both height and surface area of the black triangle increased in group A than in group B although difference was non-significant.

3.2. Histological assessment

3.2.1. H & E-stained sections

Assessment of H&E-stained sections of study groups revealed that there is no edema or hypersensitivity reactions in the injected tissues. Neither inflammatory response nor granulomatous foreign body reaction was observed in the gingival tissues **(Fig.4).** The control group showed few collagen fibers in the submucosal tissues together with few fibroblasts and minimally detected blood capillaries. A significant increase in number of fibroblasts and endothelial lined- spaces was observed in group Ia and group Ib. Group II showed better tissue response, which was characterized by much more cellular accumulation and matrix deposition. Newly formed capillaries (angiogenesis) have populated the tissue together with proliferation of fibroblasts. Compared to the Control and hyaluronic acid groups, more capillaries were distributed in group II. Minimal change was observed in group IIb compared to group IIa.

3.2.2. Masson's trichrome stained sections

The area percentage of collagen fibers differed significantly among the groups ($P < 0.001$). Control group recorded 35.7 ± 2.53 compared 42.57± 3.37 and 37.23± 2.24 for groups Ia and Ib respectively. HA\Chitosan group showed more dense blue stained collagen fibers 60.54±2.43 at 6-month post-injection that decreased to 57.44± 3.15 after 9 months **(Fig.5).**

4. Discussion

Interdental "black triangles" were considered as the third less attractive esthetic problem after caries and crown margins. The presence or absence of the interdental papilla was and still is a great concern to periodontists and patients [20]. Several surgical approaches using traditional periodontal plastic and augmentation procedures have been proposed to overcome this problem. However, these techniques were found to be invasive with increased patient morbidity, limited success, and long-term stability [21]. Application of non-invasive techniques such as the use of commercially available hyaluronic acid gel can replace the conventional invasive methods [22]. Hyaluronic acid has more structural and physiological functions in tissues, including extracellular and cellular interactions, interactions with "growth" factors, and regulation of osmotic pressure and tissue lubrication, which helps in maintaining the structural and homeostatic integrity of tissues [23]. Chitosan and its derivatives have excellent biocompatibility, nontoxicity to human beings, biodegradability, the reactivity of the deacetylated amino groups, selective permeability, polyelectrolyte action, antimicrobial activity, ability to form gel, film, and sponge, absorptive capacity, anti-inflammatory and wound healing [24]. The aim of this study was to evaluate and compare clinical and histological effects of cross-linked hyaluronic acid gel injection versus hyaluronic acid and chitosan hydrogel in management of interdental papilla deficiency. Clinical results showed that the heigh and surface area of the black triangle in both groups significantly changed from base line to six and nine months. This finding was similar to that of Becker et al. who concluded that injection of hyaluronic acid gel significantly decreased the interdental black triangle in the esthetic zone [25]. This may be related to the viscoelastic property of HA which maintains tissue structure, volume, rigidity, and gap filling [26]. The mechanical and physical properties of the gel depend on the interaction of the constituent polymer with water [27]. HA binds with water and swells giving volume to tissues and has good water retention capability both at high and low relative humidity [28]. In accordance with our results, Lee W, et al., whose recorded a mean reduction percentage of 92.55% ± 13.46% after 6 months. And they demonstrated that interdental papilla reconstruction using an injectable hyaluronic acid gel can be a viable treatment option for interdental papilla deficiencies in small areas [29]. At 9 months, both groups showed increase in the height of the black triangle and increase in the surface area. Comparing group A (HA) with group B (HA and chitosan) revealed that the changes were obvious in group A than group B and it may be related to that Hyaluronic acid degrades naturally in the body and, therefore, the duration of maintenance of the injectable hyaluronic acid gel is critical. Although difference was non-significant, it proves that Hydrogel acquired from chitosan-hyaluronic can be used to create scaffolds that are used to support osteoblasts, cementoblasts and support the periodontal fibroblast cells, as well as used for periodontal tissue engineering [30]. Regarding histological examination, Group I showed an increase in area percentage of collagen fibers compared to control group as hyaluronic acid promotes angiogenesis and migration of mesenchymal stem cells [31, 32]. The total area occupied by collagen decreased in group Ib compared to group Ia and this could be explained based on the biological degradation of hyaluronic acid. Contrarily, minimal decrease was observed between group IIa and group IIb. That may be due to the increased vascularity and cellularity in these groups that have been affected by the prolonged stability of hyaluronic acid.

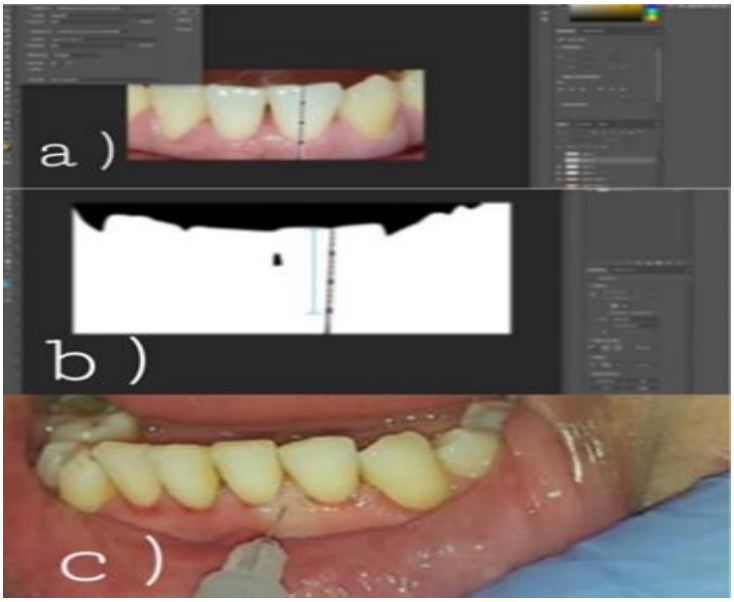

Figure 1: a) the clinical photograph was imported to photoshop. **b)** turning the area of the black triangle into black while the rest of the image turned white. **c)** drug injection.

Table 1: Mean and Standard deviation (SD) values for clinical parameters for both groups

Clinical Parameters	Group (A) Hyaluronic acid	Group (B) Hyaluronic acid & Chitosan hydrogel	p-value
Plaque index			
Base line	1.07 ± 0.57	1.12 ± 0.39	0.545
6 months	0.53 ± 0.45	0.49 ± 0.23	0.451
9 months	0.48 ± 0.33	0.41 ± 0.19	0.067
bleeding index			
Base line	1.10 ± 0.17	1.17 ± 0.20 0.75±0.18	0.633
6 months	0.62± 0.21	0.49±0.23	0.478
9 months	0.58± 0.14		0.361
Probing depth (mm)			
Base line	3.76 ± 0.45	3.37 ± 0.59	0.506
6 months	3.23 ± 0.64	3.35 ± 0.32	0.638
9 months	3.19 ± 0.35	3.26 ± 0.46	0.269
Height of the black triangle (mm)			
Base line			
6 months	2.13±0.40	2.21±0.45	0.352
9 months	1.03±0.17	0.98±0.45	0.285
	1.39±0.34	1.18±0.47	0.224
Surface area of the black triangle :(mm2)			
Base line			
6 months	0.68±0.42	0.76±0.35	.0476
9 months	0.36±0.24	0.31 ±0.11	0.145
	0.49±0.07	0.37±0.07	0.274

*Significant ($p \leq 0.05$)

Table 2: Mean and Standard deviation (SD) values for area percentage of collagen fibers for different groups

	Control (n=25)	Group I		Group II	
		Group Ia (n=25)	Group Ib (n=25)	Group IIa (n=25)	Group IIb (n=25)
Area percentage of collagen fibres	35.7 ± 2.53	42.57± 3.37	37.23± 2.24	60.54±2.43	57.44± 3.15

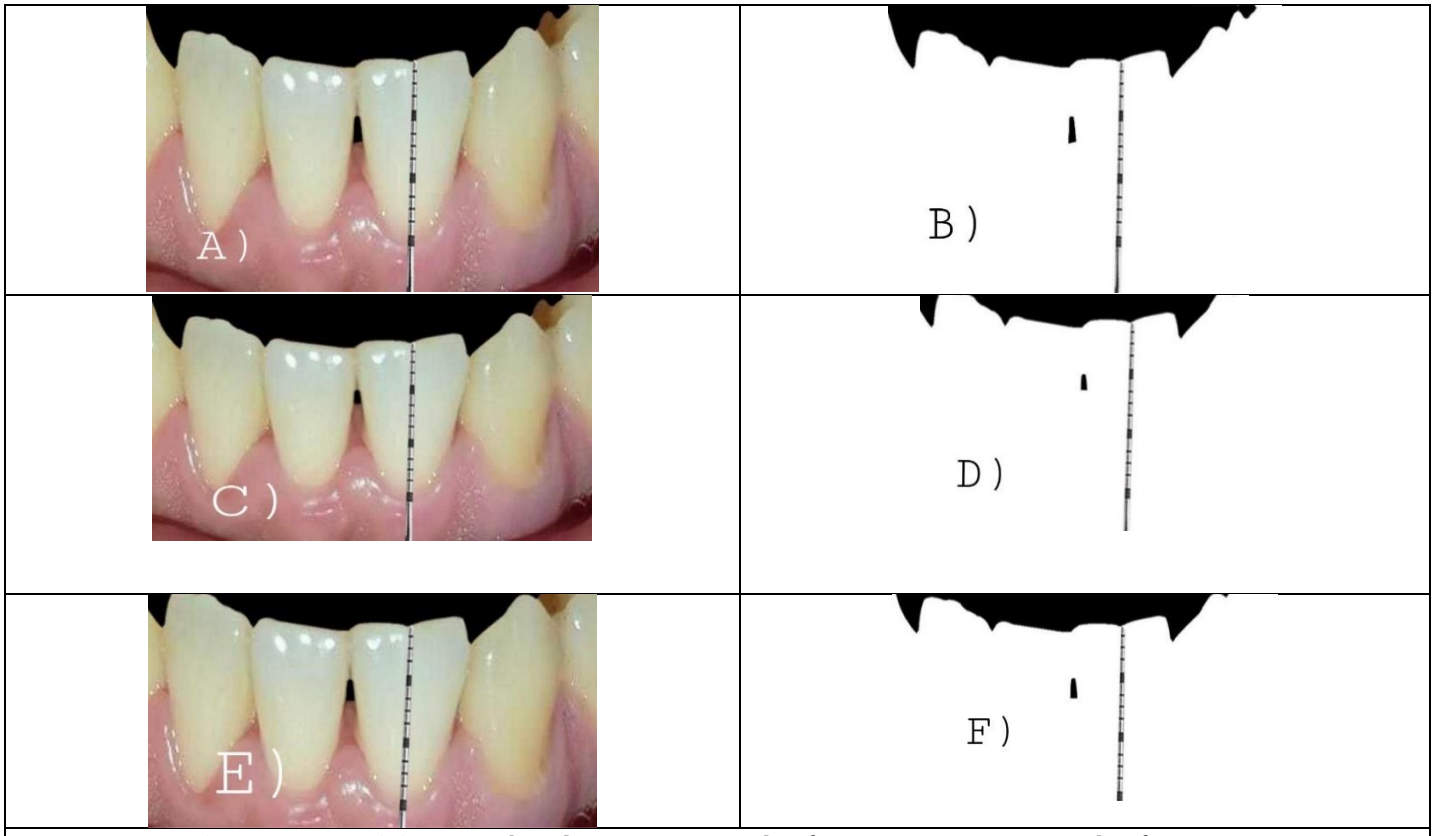

Figure 2: Group (A): A & B: base line. C&D: 6 months after injection. E&F: 9 months after injection.

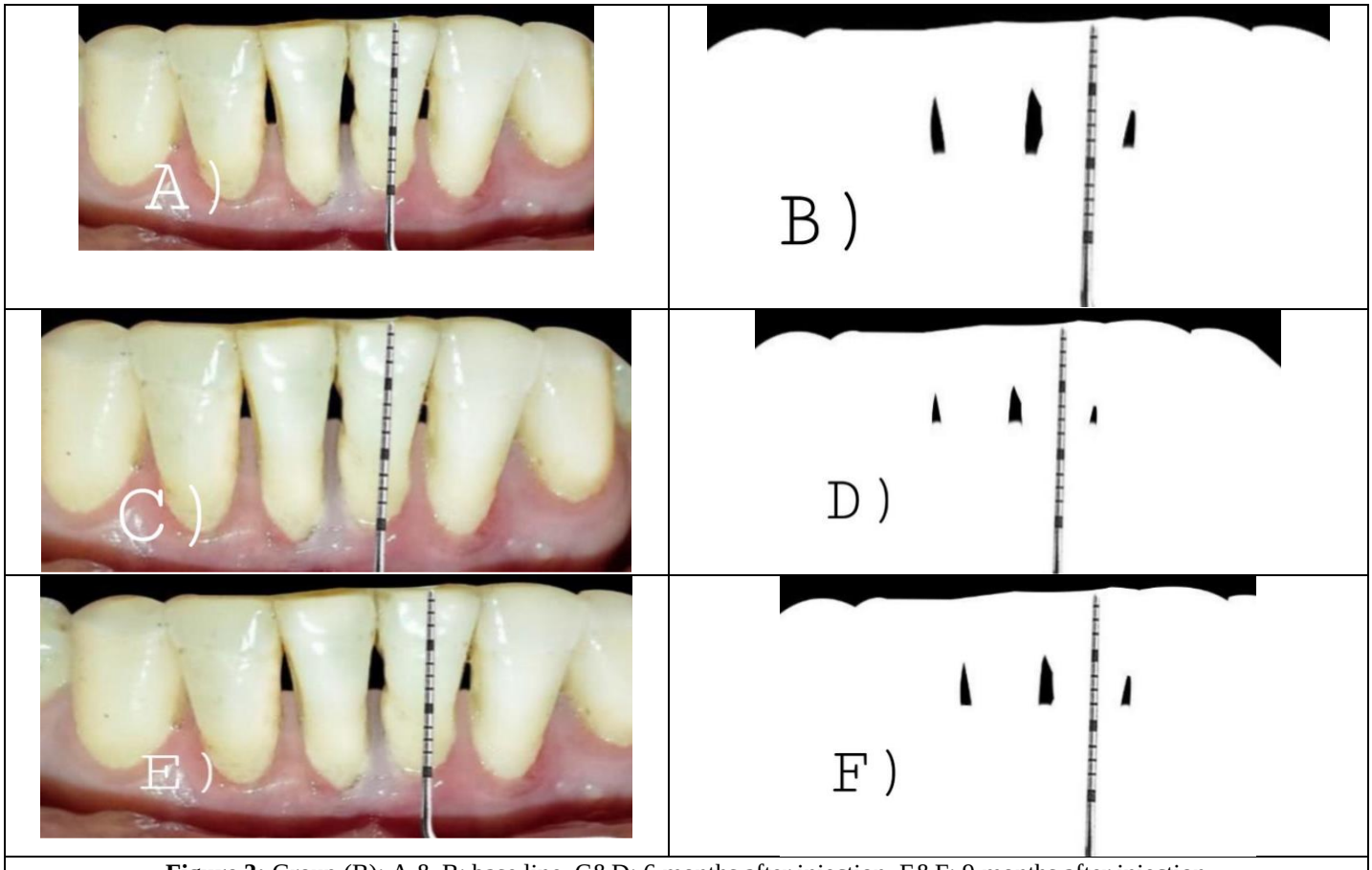

Figure 3: Group (B): A & B: base line. C&D: 6 months after injection. E&F: 9 months after injection

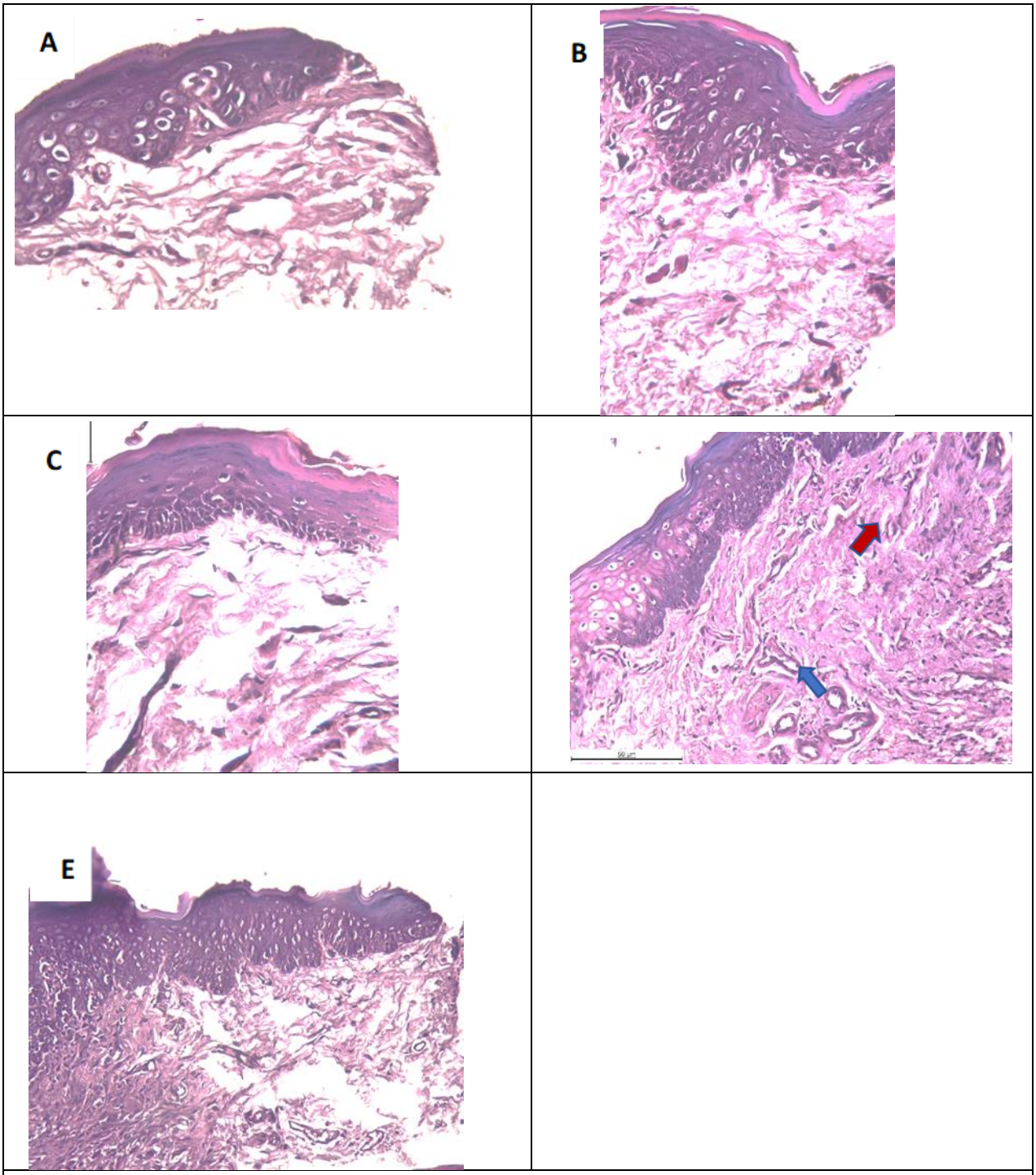

Figure 4:H&E-stained sections of different group.A)Control group. B)GroupIa 6 months post-injection of HA gel.C)Group Ib 9 month post-injection of HA gel. D)Group IIa 6 month post-injection of HA\Chitosan hybrid. E) Group IIb 9 month post-injection of HA\Chitosan hybrid. Red arrow refers to fibroblast. Blue arrow refers to blood caoillaries (40X).

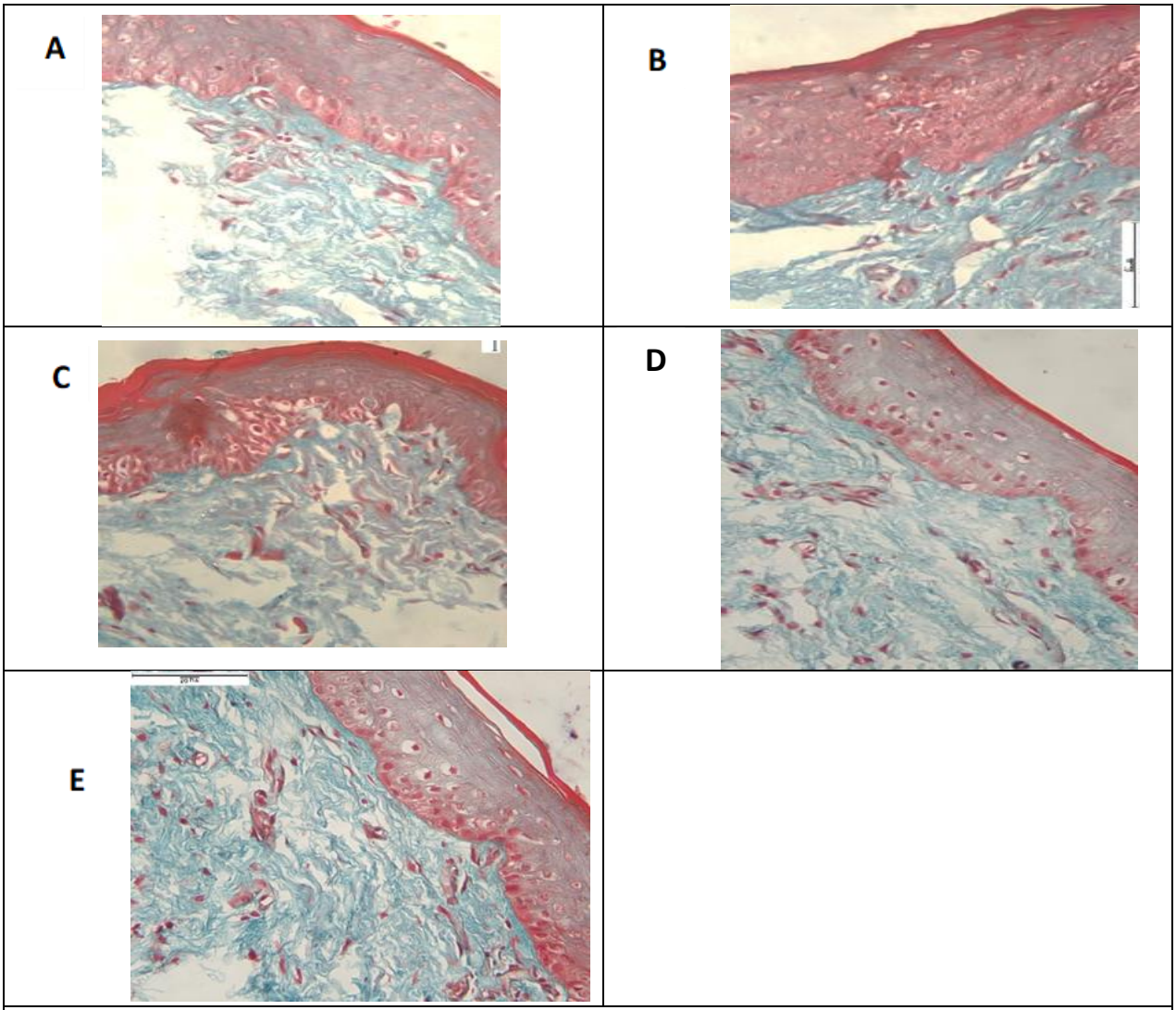

Figure 5: Masson trichrome stained sections of different group.A)Control group. B)GroupIa 6 months post-injection of HA gel.C)Group Ib 9 month post-injection of HA gel. D)Group IIa 6 month post-injection of HA\Chitosan hybrid. E) Group IIb 9 month post-injection of HA\Chitosan hybrid (40 X).

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