Culture of Human Gingival Fibroblasts on a Biodegradable Scaffold and Evaluation of Its Effect on Attached Gingiva: A Randomized, Controlled Pilot Study

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Background: An adequate width of attached gingiva is necessary to maintain healthy periodontium, especially in orthodontics or restorative treatments in periodontics. The purpose of this study was to evaluate the width of attached gingiva after clinical application of a cultured gingival graft compared to a periosteal fenestration technique.

Methods: This study was conducted on nine patients (18 sites) with insufficient attached gingiva adjacent to at least two teeth in contralateral quadrants of the same jaw. A small portion ($\sim 3 \times 2 \times 1$ mm) of attached gingiva (epithelial + connective tissue) was removed with a surgical blade. After culture of gingival fibroblasts, 2×10^5 cells in 250 μl nutritional medium were added to 250 μl collagen gel. One tooth in each patient was randomized to receive a periosteal fenestration technique for gingival augmentation (control) or a tissue-engineered mucosal graft (test). Clinical parameters measured at baseline and 3 months included width of keratinized tissue, probing depth, and width of attached gingiva.

Results: An increased amount of keratinized tissue was seen at all treated sites after 3 months. The mean increased amount of attached gingiva was 2.8 mm at test sites and 2 mm at control sites; this difference was significant ($P < 0.05$).

Conclusion: Based on the results of this investigation, the tissue-engineered mucosal graft is safe and capable of generating keratinized tissue. *J Periodontol 2007;78:1897-1903.*

KEY WORDS

Gingiva; tissue engineering.

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The establishment of an adequate zone of attached gingiva is one of the goals of periodontal surgery.[1] Attached gingiva was created first by denuding an extensive area of alveolar bone; however, this procedure resulted in protracted and painful postoperative periods and some loss of crestal bone.[2,3] Periosteal retention and periosteal fenestration procedures also have been used to create gingiva, but they were reported to be unpredictable.[4-6] With the continued refinement of mucogingival surgery, emphasis has been placed on conserving and repositioning the existing gingiva; therefore, the apically positioned flap was introduced.[7] Currently, soft tissue augmentation procedures are performed using the patient's masticatory mucosa (connective tissue[8] or free gingival graft[9]) as donor material because of the predictability of this procedure. However, mucosal grafts have disadvantages: limitation in the size of a donor site, postoperative pain, increased morbidity, compromised esthetic outcome, formation of exostosis, and increased procedure time if a surgical complication, such as rupturing a palatal blood vessel, arises.[10,11] For these reasons, the patient and clinician have been interested in an alternate source for donor

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tissue. Acellular dermal matrix allograft[§] has been used in periodontal, plastic, and reconstructive surgery since 1994.[12] Acellular dermal matrix allograft can be used for soft tissue augmentation procedures without procuring donor tissues from the patient's own palate. Recently, there has been substantial and growing public and scientific awareness of a relatively new field of applied biologic research called tissue engineering. This field builds on the interface between materials science and biocompatibility and integrates cells, natural or synthetic scaffolds, and specific signals to create new tissues.[13] The primary goal of tissue engineering is to create material constructs that improve the quality of healing and/or supplement or replace malfunctioning tissues and organs. Such constructs usually consist of one or more cell types in contact with a non-cellular scaffold that provides physical support for the cells and establishes functional micro- and macroarchitecture.[14] Scaffolds are made from a variety of synthetic polymers and from type I collagen, which is minimally antigenic and is a natural substrate for cell adhesion and growth.[15] Certain growth factors that are necessary for cell survival and function, e.g., basic fibroblast growth factor, bind to collagen and are released from it in a controllable manner with a minimal loss of activity.[16,17] Collagen is bioabsorbable and is cleaved into peptides that are non-toxic to cells.[14] A few studies[18-24] discussed the clinical use of cultured mucosal grafting for intraoral mucosal defects. Cultured epithelium fabricated with living mucosal cells has proved to be a good grafting material for any type of mucosal defect, such as peri-implant soft tissue difficiency.[19] The tissue-engineered gingival grafts containing fibroblasts also were used successfully for gingival augmentation.[20,21,24] An obvious advantage of using tissue-engineered cell sheets is that a very small tissue sample from a donor site can serve as source material to cover a large recipient site.[21,24] The aim of this randomized, controlled, within-patient paired design study was to evaluate the width of attached gingiva after clinical application of cultured gingival graft compared to a periosteal fenestration technique.

MATERIALS AND METHODS

Nine patients (18 sites), eight females and one male aged 20 to 48 years, with insufficient attached gingiva adjacent to at least two teeth in contralateral quadrants of the same jaw were selected from patients referred to the Periodontology Department, Dental School, Shaheed Beheshti University. From these nine patients, eight patients needed to undergo prosthetic rehabilitation, whereas one patient needed orthodontic therapy. This study was conducted between July 2005 and December 2006.

The criteria for inclusion in the study were no systemic diseases that contraindicated periodontal surgery; no medications affecting periodontal surgery; no pregnancy or lactation; no periodontal diseases; no smoking; full-mouth plaque score and full-mouth bleeding score <20% at the time of the surgical procedures; and at least two non-adjacent teeth with an insufficient zone of attached gingiva that required soft tissue grafting.

The patients were informed of the purpose of the study and were required to give informed consent. The study design and consent form were approved by the ethical committee of the School of Medical and Dental Sciences, Shaheed Beheshti University.

Measurements

Full-mouth plaque and bleeding scores, probing depth (PD), and width of keratinized and attached gingiva were recorded for the involved teeth at baseline and 3 months after surgery. The width of keratinized gingiva (the distance between the gingival margin and the mucogingival line) was measured to the nearest millimeter with a Williams periodontal probe. The mucogingival line was detected with the Roll test, and Schiller's solution was used for verification. The amount of attached gingiva was determined by computing the distance from the free gingival margin to the mucogingival junction and then subtracting the PD in the midbuccal site for each involved tooth.

This study was done with three clinicians. Two clinicians, who were calibrated prior to the study and masked about the surgical technique, measured the clinical parameters (PD and width of keratinized and attached gingiva) at baseline and 3 months after surgery. The surgery was done by the third clinician.

Biopsy

On the first visit, a biopsy of attached gingiva was performed. After local anesthesia, ~3 × 2 × 1-mm area of attached gingiva (epithelial + connective tissue) was removed with a surgical blade (Fig. 1). The sample was put in a nutritional medium (Roswell Park Memorial Institute [RPMI] 1640[‖]) containing antibiotics (penicillin, 100 IU/ml,[¶] and streptomycin, 100 μg/ml[#]) and transferred to the laboratory. The patients were instructed to use chlorhexidine digluconate 0.2% mouthwash for several days.

Culturing Technique

On arrival at the laboratory, National Cell Bank of Iran, Pasteur Institute of Iran, the tissue was rinsed in phosphate buffered saline (PBS)[**] and transferred into a petri dish. The gingival tissue sample was cut into

§ Lifecell, Branchburg, NJ.
‖ Gibco, Paisley, Scotland, U.K.
¶ Sigma.
# Sigma.
** Sigma.

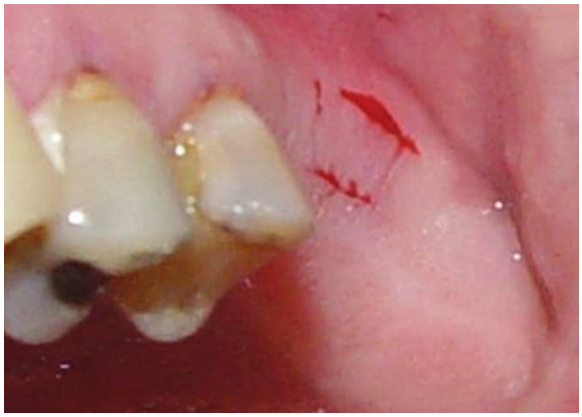

Figure 1.
A 3 × 2 × 1-mm area of attached gingiva is biopsied to culture fibroblasts.

Figure 2.
Collagen scaffold containing the patient's cultured fibroblasts after 8 days.

small pieces and treated with a 0.25% trypsin solution.[††] After incubation at 37°C for 1 hour, the epithelial layer was peeled off from the connective tissue gently and rinsed with phosphate buffered salino (PBS). Human gingival fibroblasts were obtained by overnight digestion of gingival connective tissue with a solution of 80 μl/ml type I collagenase[‡‡] at 37°C, 5% CO_2. Fibroblasts were cultured in nutritional medium (RPMI 1640) containing 10% AB human serum[§§] and antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml). During this stage, the culture medium was renewed twice a week. When the culture reached 80% to 90% confluence for fibroblasts, the cells were detached and used to produce tissue-engineered gingival graft.

Engineered gingival lamina propria was produced by mixing 250 μl bovine skin collagen type I[‖‖] with 250 μl nutritional medium (RPMI 1640) containing human gingival fibroblasts (2×10^5). The mixture was poured into a well of a six-well plate. Initial polymerization of bovine skin collagen containing fibroblasts was performed at room temperature for 10 minutes in a laminar flow hood. Then, this mixture was incubated until completely polymerized at 37°C, 5% CO_2 for 30 minutes. Tissue was grown in culture medium (RPMI 1640) containing 10% AB human serum and antibiotics. On the eighth day after fibroblast seeding, collagen scaffold containing the patient's cultured fibroblasts was rinsed in PBS several times to remove the AB human serum.

The tissue-engineered gingival graft (Fig. 2) was transferred to the dental clinic in a sterile six-well plate, filled with nutritional medium without serum, and sealed with parafilm.

Surgical Technique and Follow-Up

Test and control sites for the patients were determined randomly by a coin toss. At the control site, after local anesthesia, a horizontal incision was made at the mucogingival junction (submarginal), and vertical incisions were extended apically for ~10 mm. Sharp dissection was done with a #15 knife. Approximately 7 mm from the coronal incision, a periosteal fenestration was done with a #15 knife. After preparation of a partial-thickness recipient bed, a gauze[¶¶] in an appropriate size was placed on the recipient bed, and then an aluminium foil with an appropriate size was adapted on the recipient bed. These components were fixed with circumferential and interdental sutures (Fig. 3).

At the test site, preparation of the recipient bed was similar to the control site; however, after preparation of the partial-thickness recipient bed, the tissue-engineered gingival graft was removed from the sterile package, shaped, and adapted to the recipient site. A gauze in an appropriate size was placed on the graft surface, and then an aluminium foil with an appropriate size was adapted on the recipient bed. These components were fixed with circumferential and interdental sutures (Fig. 4).

Chlorhexidine digluconate 0.2% mouthwash was not prescribed during the first 2 weeks to avoid damage to the fibroblasts, and toothbrushing was discontinued. Sutures were removed 2 weeks following the surgery. Supragingival professional tooth cleaning, along with oral hygiene instructions, were performed weekly for the first 6 weeks post-surgery and then once a month for up to 6 months post-surgery.

Statistical Analysis

The normal distribution of the collected data was tested by the one-sample Kolmogorov-Smirnov test. The paired *t* test was used to compare the results

†† Sigma.
‡‡ Sigma.
§§ Iran Blood Transfusion Organization, Tehran, Iran.
‖‖ INAMED, Santa Barbara, CA.
¶¶ Abzar Darman, Tehran, Iran.

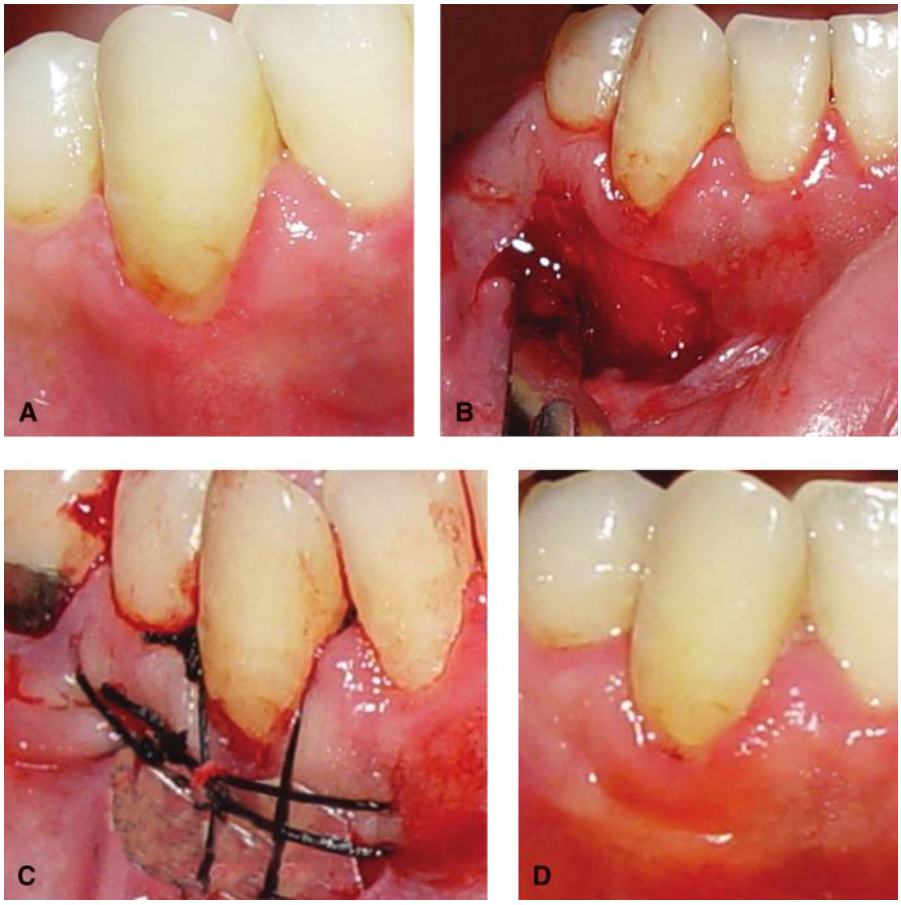

Figure 3.
Control site. ***A)*** *Preoperative photograph.* ***B)*** *Periosteal fenestration technique is performed.* ***C)*** *A gauze is placed on the recipient bed, and then a foil with an appropriate size is placed on the gauze and fixed with circumferential and interdental sutures.* ***D)*** *At 3 months, tissue then an aluminium foil with an appropriate size was adapted on the recipient bedaugmentation is obtained.*

before and after surgery in the control and test groups and between the groups for normally distributed data. The Wilcoxon signed-rank test was used for data that were not normally distributed.

RESULTS

The patients had mild pain for 2 or 3 days after surgery. Three months later, biopsies were performed on four patients to evaluate the histologic characteristics at control and test sites. The histologic results at the control and test sites were similar. The histologic features demonstrated a fully keratinized tissue. The gingival epithelium was acanthotic, and parakeratotic and anastomosed rete-pegs were seen. Lamina propria was a fibrovascular tissue with infiltration of mononuclear inflammatory cells (Fig. 5).

The clinical parameters, including PD, width of keratinized gingiva, and width of attached gingiva, were recorded 3 months after surgery. PD, width of keratinized gingiva, and width of attached gingiva at baseline and 3 months after surgery are reported in Table 1; the comparison of post-surgery clinical parameters between the test and control groups is shown in Table 2. These results showed that the mean width of keratinized and attached gingiva had increased 2.8 mm in test sites and 1.9 and 2 mm in control sites, respectively, at 3 months after surgery. The difference between the width of keratinized gingiva in test and control sites was significant ($P < 0.05$). The difference in the width of attached gingiva was nearly significant ($P < 0.06$; Table 2). The comparison of the widths of the keratinized and attached gingiva before and after surgery showed that the differences were significant in each group ($P < 0.00$; Table 1).

DISCUSSION

Recent developments in biomedical engineering as well as basic biology and medicine has enabled us to induce cell-based regeneration of body tissue to self-repair defective tissue or substitute biologic functions of damaged organs. The most successful application of cell transplantation involves the development of a tissue-engineered skin equivalent. Skin tissue is needed to treat burn victims and patients with diabetic ulcers.[25] This need led to early research on the engineering of skin tissue and resulted in the first United States Food and Drug Administration–approved tissue-engineered products for clinical use.[26,27] In oral surgery, cultured gingival keratinocyte grafts have been applied to cover epithelial defects in preprosthetic surgery. These transplants usually are cultured according to the technique of Rheinwald and Green,[28] with a feeder layer of γ-irradiated 3T3 mouse fibroblasts. A new technique of soft tissue management for implant therapy uses a cultured epithelial graft. This technique has a problem related to the mechanical weakness of cultured epithelium. Cultured epithelium possesses only the epithelial layer, and it is too thin in epithelial sheets to tolerate mechanical trauma during the healing period.[19]

In the present study, we used gingival fibroblasts and a bioabsorbable scaffold because it has been demonstrated that the keratinization of gingival

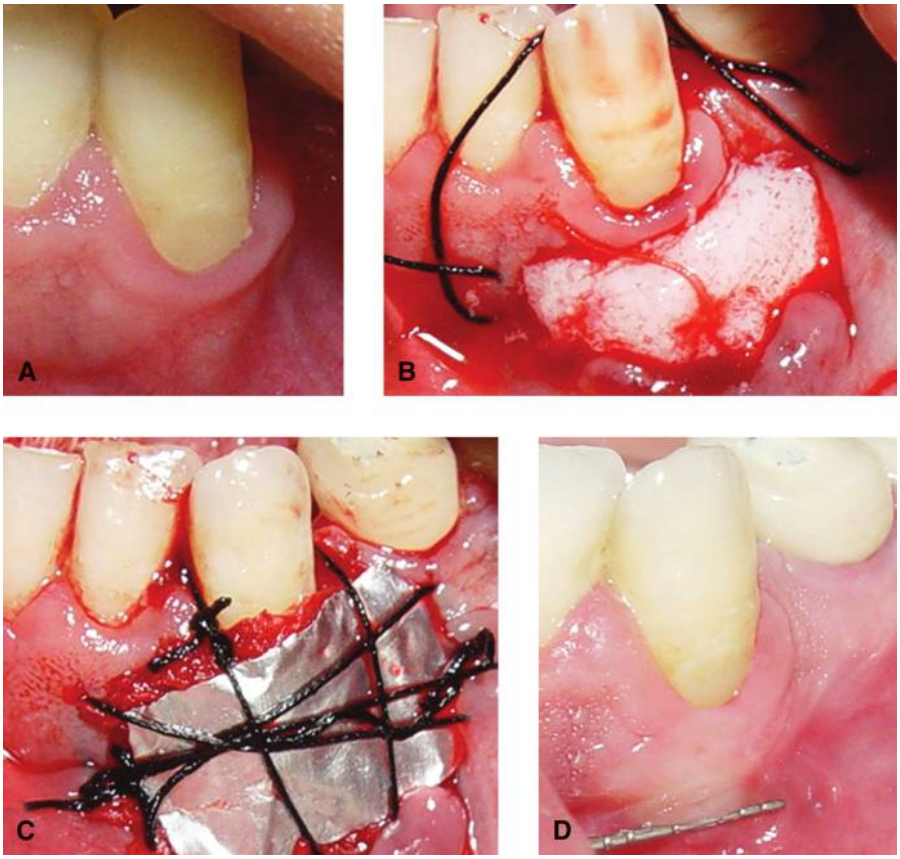

Figure 4.
Test site. ***A)*** *Preoperative photograph.* ***B)*** *After preparation of the bed, a tissue-engineered gingival graft is adapted to the recipient area.* ***C)*** *A gauze is placed on the recipient bed, and then a foil with an appropriate size is placed on the gauze and fixed with circumferential and interdental sutures.* ***D)*** *At 3 months, tissue augmentation is obtained.*

epithelium is controlled by morphogenetic stimuli of the underlying connective tissue[29] and because of easier handling of this kind of tissue-engineered graft. The present study showed an increased amount of keratinized tissue on all treated sites after 3 months. For the test group, the average of the increased amount of keratinized tissue in the middle site was 2.8 mm, which was statistically significant ($P <$ 0.00). At control sites, the mean of the increased amount of keratinized tissue was 1.9 mm, which was statistically significant ($P < 0.00$). The comparison between test and control groups showed that the width of keratinized and attached gingiva increased clinically (0.9 mm and 0.8 mm, respectively), and statistically significant differences were found 3 months after surgery for keratinized gingiva ($P <$ 0.05).

Pini Prato et al.[21] introduced an autologous cell hyaluronic acid graft for gingival augmentation in mucosal surgery. They published a case series[20] that used gingival fibroblasts seeded onto the benzyl ester of a hyaluronic acid scaffold. The prepared tissue was transferred to their clinic after 8 days and then grafted onto the periosteal bed. An increased amount of keratinized tissue on all treated sites was reported after 3 months. The increased amount of keratinized tissue was 2.00 ± 0.41 mm in the mid-buccal site. The histologic findings of Pini Prato et al.[20,21] showed a keratinized epithelium with a dense connective tissue resembling the results observed in the present study.

The safety and effectiveness of a tissue-engineered skin equivalent, a living human fibroblast-derived dermal substitute (HF-DDS), was evaluated and compared to a gingival autograft (GA) consisting of donor tissue harvested from the patient's palate in a procedure designed to increase the amount of keratinized tissue around teeth that did not require root coverage.[24] McGuire and Nunn[24] used a bioabsorbable polyglactin scaffold and allogenic dermal fibroblasts. For the test group, the mean of increased keratinized tissue was 2.72 mm 3 months after surgery. The GA generated more keratinized tissue and shrank less than the HF-DDS graft, but the test graft generated tissue that appeared more natural. Similar histologic results were reported in that study and the present study, despite the use of different fibroblasts (gingival and skin fibroblasts). Carranza and Carraro[30] evaluated the effect of periosteal fenestration in a gingival extension operation. They reported an increase of 2.3 ± 0.38 mm in the width of keratinized tissue with that technique. Bowers[31] showed a 2.3-mm increase in the width of keratinized tissue using periosteal fenestration. Also, Allen and Shell[32] reported an increase of 2.3 mm in the width of keratinized tissue after 40 weeks with periosteal fenestration. In the present study, the mean increased keratinized tissue at the control sites was 1.9 mm, which was approximately similar to the results of the aforementioned studies. The histologic findings at the control sites in the present study (a parakeratinized epithelium with a dense connective tissue) were similar to those in the study by Carranza and Carraro.[30] Tissue-engineered gingival grafts have several advantages: a very small donor site, sufficient amounts of keratinized tissue obtained, and minimal

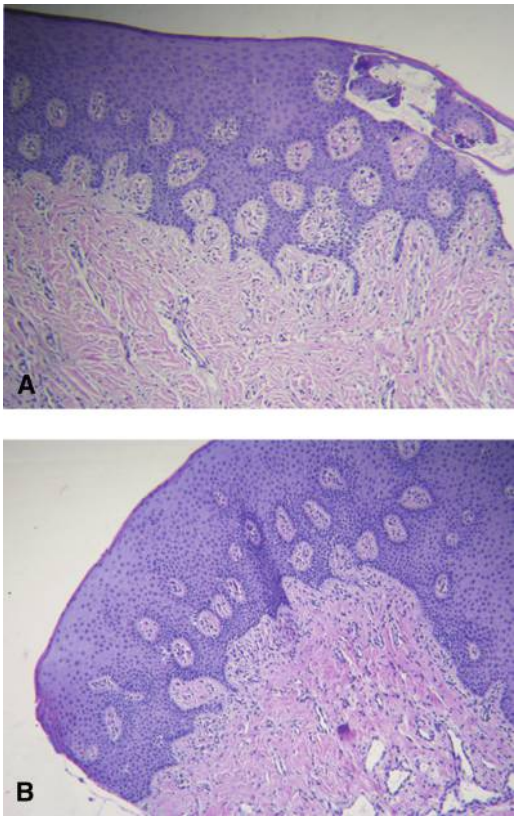

Figure 5.
Histology shows a dense keratinized tissue at control ***(A)*** *and test* ***(B)*** *sites (hematoxylin and eosin; original magnification ×100).*

Table 1.

Clinical Parameters (mm) at Baseline and Post-Surgery

Parameter	Baseline (mean ± SD)	Post-Surgery (mean ± SD)	*P* Value
PD			
Test	1.3 ± 0.5	1.2 ± 0.4	<0.17
Control	1.1 ± 0.3	1.1 ± 0.3	<0.35
Width of keratinized gingiva			
Test	1.3 ± 0.4	4.1 ± 1	0.00
Control	1.5 ± 0.4	3.4 ± 0.8	0.00
Width of attached gingiva			
Test	0.2 ± 0.4	3 ± 1	0.00
Control	0.3 ± 0.4	2.3 ± 0.7	0.00

discomfort for the patient. The results of this study showed that it is possible to generate keratinized tissue with these tissue-engineered materials; however, further controlled clinical testing is needed.

Table 2.

Comparison of Post-Surgery Clinical Parameters (mm)

Parameter	Post-Surgery (mean ± SD)		*P* Value
	Test	Control	
PD	1.2 ± 0.4	1.1 ± 0.3	<0.35
Width of keratinized gingiva	4.1 ± 1	3.4 ± 0.8	<0.05
Width of attached gingiva	3 ± 1	2.3 ± 0.7	<0.06

CONCLUSIONS

The purpose of this randomized, controlled clinical trial was the culture of human gingival fibroblasts on a bioabsorbable scaffold and evaluation of its effect on attached gingiva. The comparison between test and control groups showed that the width of keratinized and attached gingiva increased clinically (0.9 and 0.8 mm, respectively); however, statistically significant differences were found only for keratinized gingiva 3 months after surgery. Based on the results of this investigation, the tissue-engineered gingival graft is safe and capable of generating keratinized tissue without the morbidity and potential clinical difficulties associated with donor site surgery.

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