Human gingival fibroblasts culture in an autologous scaffold and assessing its effect on augmentation of attached gingiva in a pilot clinical trial

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Original Article

Abstract

BACKGROUND AND AIM: An important goal of periodontal plastic surgery is the creation of attached gingiva around the teeth. In this study, the aims were to culture gingival fibroblasts in a biodegradable scaffold and measure the width of attached gingiva after the clinical procedure.

METHODS: This study was carried out on 4 patients (8 sites), with inadequate attached gingiva next to at least two teeth in contralateral quadrants of the same jaw. A biopsy of attached gingiva (epithelial + connective tissue) was taken using a surgical blade. Following culture of gingival fibroblasts, 250×10^3 cells in 250 µl nutritional medium were mixed with platelet-rich in growth factor (PRGF). Periosteal fenestration technique was done on one side (control) and tissue-engineered mucosal graft (test) was carried out on the contralateral side in each patient. The width of keratinized tissue, probing depth (PD) and width of attached gingiva were recorded at baseline and 3 months after the operation.

RESULTS: An increased width of keratinized and attached tissue on all operated sites after 3 months was observed. These results showed the increased mean of the width of keratinized and attached gingiva to be 4.17 mm and 4.14 mm in test and 1.10 mm and 1.10 mm in control sites, respectively. The difference of keratinized and attached gingiva width between test and control sites was significant (P = 0.030, and P = 0.010 respectively).

CONCLUSION: According to the results of this study, PRGF can be used as a scaffold to transfer gingival fibroblasts to recipient sites with significant clinical results.

KEYWORDS: Tissue Engineering; Gingiva; Blood Platelet; Scaffold

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An important goal of periodontal plastic surgery is the regeneration of attached gingiva.[1] Several surgery techniques such as denudation,[2] periosteal retentions,[3] periosteal fenestration,[4,5] apically positioned flap,[6] connective tissue grafts,[7] free gingival grafts,[8] and acellular dermal matrix allografts[9] were proposed to increase the attached gingiva. At the present time, connective tissue and free gingival grafts are usually used to augment the gingiva, because of the predictability of these procedures. However, some disadvantages are existed about masticatory mucosal grafts such as postoperative pain at the donor site, morbidity, reducing the size of a donor site, formation of exocytosis, poor color matching to the surrounding tissue, and increased

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procedure duration.[9-11] Therefore, a technique with fewer disadvantages is preferred by the patients and clinicians. Tissue engineering technology is already being developed and applied in different medical fields to replace cartilage, bone, cardiovascular components, pancreas, and skin.[12] According to this fact, tissue engineering could also be useful in periodontology. Cells, scaffolds and growth factors are the three principal components for preparation of a tissue-engineered construct.[12] Epithelial cells and fibroblasts are used to develop the tissue engineered gingival grafts (TEGG).[13-21] Benzyl ester hyaluronic acid, collagen, polyglactin mesh, and chitosan are the scaffolds used in periodontology aiming at gingival augmentation.[15,19,20,22]

Platelet-rich plasma (PRP) is an autogenous concentration of platelets in which platelet counts should be 5 times more than the baseline number of platelets in whole blood.[23] PRP has been used clinically in human since the 1970s for its healing properties.[24] PRP is also called plasma rich in growth factors (PRGF), platelet concentrates (PCs), and autologous platelet gel (APG).[25] Platelet activation in PRP results in releasing of growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and insulin-like growth factor (IGF).[25] All of which are proven beneficial to wound healing.[26] PRP as a biogenic scaffold for tissue engineering have not been applied in the reconstruction of the oral mucosa. We used PRP as a scaffold in tissue engineering, because of the safety, low preparation cost, and existence of fibrin and growth factors in it.[26]

The aim of our research was to culture the human gingival fibroblasts in PRGF as a scaffold and investigate its influence on gingival augmentation.

Methods

This study was a pilot randomized controlled clinical trial. Four woman patients (8 sites), aged 32 to 46 years, with less than enough attached gingiva surrounding at least two teeth in contralateral quadrants of the same jaw were included in this study. They were selected from the patients referred to Department of Periodontology of Kerman Dental School, Iran.

Inclusion criteria in this study were no pregnancy and lactation, no medication affecting on periodontium, no smoking, no periodontal diseases, no systemic diseases affecting on periodontium or contraindicating periodontal surgery, full mouth plaque index and full mouth bleeding index < 20% at the time of surgery.

Explanation regarding the aim and course of the study was given to patients and informed consent was obtained. This study was ethically approved by Ethics Committee of Kerman School of Medical and Dental Sciences, Iran. Kerman University approved the consent form and design of the present study with ethical code 96/86/K. This clinical trial was registered in IRCT website with number IRCT201108135305N2.

Clinical measurements: At baseline and 3 months after surgery, probing depth (PD), the width of keratinized and attached gingiva were measured and recorded. Williams's periodontal probe was used to measure the width of keratinized gingiva from the gingival margin to the mucogingival junction to the nearest millimeter. Roll test was used for detection of mucogingival junction. The PD was subtracted from the width of keratinized gingiva, and the width of attached gingiva was specified in the mid-buccal region for each involved teeth. One clinician who was blind to this study measured all of the clinical parameters at baseline and 3 months after surgery and also allocated surgery sites. Another clinician who was not blind to the study performed all of the surgical procedures.

Biopsy and cell culturing: At the first visit, a biopsy (~ 3 × 2 × 1 mm) was taken from a site with adequate attached gingiva under local anesthesia. The gingival biopsy (connective tissue and epithelium) in a

nutritional medium [Roswell Park Memorial Institute (RPMI) 1640], (Gibco, Paisley, Scotland, UK) containing antibiotics [penicillin (Sigma- Aldrich, USA), 100 IU/ml, and streptomycin (Sigma- Aldrich, USA), 100 µg/ml] was sent to the laboratory. The patients were required to use chlorhexidine digluconate 0.2% mouthwash for several days.

Each gingival biopsy was washed three times in phosphate buffered saline (PBS) (Sigma- Aldrich, USA), and then transferred to a Petri dish. A scalpel was used to cut each sample into small pieces under sterile conditions. These pieces in a petri dish containing 0.25% trypsin (Sigma- Aldrich, USA) were incubated at 37 °C, 5% CO_2 for 1 hour. After incubation, the gingival epithelium was separated from the gingival connective tissue and washed with PBS. A solution of 80 µl/ml type I collagenase (Sigma- Aldrich, USA) was added to digest the gingival connective tissue pieces in order to obtain gingival fibroblasts. After one night incubation, RPMI 1640 was added to the Petri dish and the suspension was centrifuged for 5 minutes to eliminate the collagenase enzyme. The fibroblasts were cultured in nutritional medium (RPMI 1640) containing 10% fetal bovine serum (FBS) (Gibco, Paisley, Scotland, UK), and antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml). The cells were cultured in a CO_2 incubator at 37 °C and the nutritional medium was changed twice a week. When the culture reached 80-90% confluence for fibroblasts, trypsin-EDTA was used to detach the cells for 5 minutes. Then, detached fibroblasts were used to produce tissue engineered gingival graft (TEGG).

Preparation of TEGG: PRGF was used as a scaffold for production of TEGG. For the preparation of PRGF, 10 ml blood was taken from the patients. The blood was poured into two 5 ml tubes containing trisodium citrate 3.8% (BTI, Vitoria, Spain) and was centrifuged at 2600 rpm for 5 minutes. 0.5 ml of plasma which was in the red blood cell (RBC) layer (PRGF) in each of the tubes was gathered with a micropipettor. 1 ml PRGF was poured into a 5 ml Petri dish. For activation of platelets in PRGF and neutralization of the effect of trisodium citrate, 50 µl 10% calcium chloride (BTI, Vitoria, Spain) was added. Then, 250×10^3 cells in 250 µl RPMI 1640 medium (count with hemocytometer) was added to PRGF. In order to set initially, it was put under the hood at room temperature for 10 minutes. After that, the TEGG was incubated for 30 minutes. After incubation, nutritional medium RPMI 1640 was added and incubated overnight to use in the clinic. Before transferring the graft to the clinic, it was rinsed with PBS several times (Figure 1). It was transferred to the dental clinic in a sterile situation, filled with RPMI 1640, and sealed with parafilm.
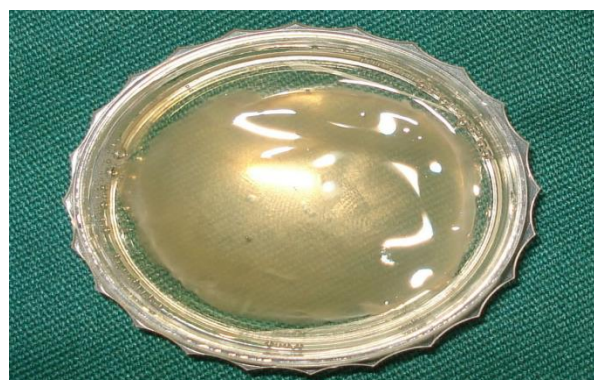
Figure 1. Plasma rich in growth factors (PRGF) containing the patient's cultured fibroblasts after 24 hours

Surgery and postoperative instructions: In this study, control (no graft) and test (TEGG) sites were selected randomly by a coin toss in each patient (simple randomization). The recipient bed preparation in control and test sites was the same. In order to prepare the recipient bed, after a local anesthesia with lidocaine 2% and epinephrine 1/80000, the mucogingival junction was horizontally incised (a submarginal incision) and two vertical releasing incisions were created apically for ~ 10 mm. The length of horizontal incision was about 15 mm. The bed was prepared by sharp dissection with a scalpel. Then, a periosteal fenestration was done about

7 mm apical to the horizontal incision. At the control site, on the partial thickness prepared bed, a gauze and aluminum foil in an appropriate size were placed on the bed, respectively. These components were stabilized with a 4-0 silk criss-cross horizontal mattress suture (Figure 2).
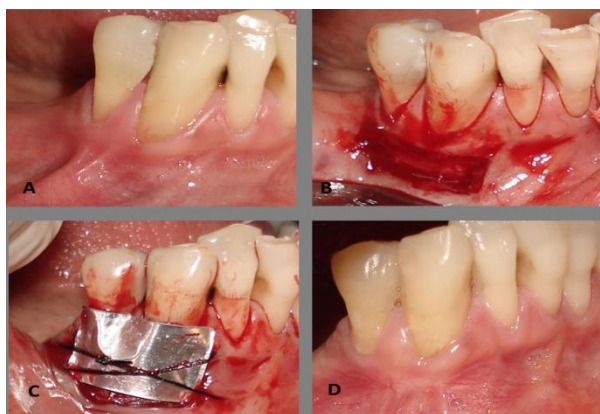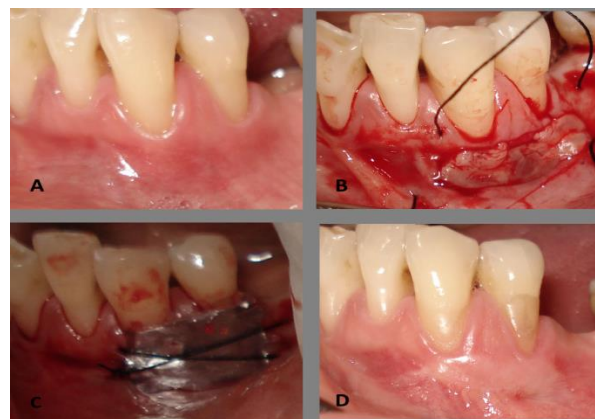

Figure 2. Control site A) Preoperative photograph, B) Periosteal fenestration technique is performed, C) A Vaseline gauze is placed on the recipient bed and a foil is adapted and fixed with a criss-cross horizontal mattress suture and D) At 3 months, tissue augmentation is obtained

At the test sites, after the TEGG was cut to an appropriate size, it was placed on the partial thickness bed. Gauze and aluminum foils in an appropriate size were adapted on the surface of the graft, respectively.[20] A 4-0 silk criss-cross horizontal mattress suture was used to fix these components (Figure 3).

During the first two weeks, the patients were asked not to use chlorhexidine mouthwash to prevent damage to the fibroblasts and discontinue toothbrush. After 2 weeks following surgery, sutures were removed. After the surgery, supragingival

tooth cleaning along with oral hygiene instructions were carried out once a week for the first 6 weeks and continued as once a month for up to 3 months post-surgery.

Figure 3. Test site A) Preoperative photograph, B) After preparation of bed tissue-engineered gingival graft is adapted to the recipient area, C) Vaseline gauze and foil are adapted and fixed with a criss-cross horizontal mattress suture and D) At 3 months, tissue augmentation is obtained

One-sample Kolmogorov-Smirnov test was used to check the normal distribution of data. Because of the abnormal distribution of the data, Wilcoxon signed-rank test was utilized to compare the results before and after the surgery in the control and test groups.

Results

The results of this study revealed that the healing process took place without any complication in both control (4 sites) and test (4 sites) sites.

The data related to the probing pocket depth, the width of keratinized gingiva and width of attached gingiva at baseline and 3 months after surgery are presented in table 1.

Table 1. Baseline and post-surgery clinical parameters

Clinical parameter		Baseline (mm) (mean ± SD)	Post-surgery (mm) (mean ± SD)	P*
Probing pocket depth	Test	1.00 ± 0	1.00 ± 0	-
	Control	1.00 ± 0	1.00 ± 0	-
Width of keratinized gingiva	Test	0.83 ± 0.75	5.00 ± 0.63	0.001
	Control	1.50 ± 0.54	2.60 ± 1.50	0.030
Width of attached gingiva	Test	0.16 ± 0.40	4.30 ± 0.51	0.001
	Control	0.50 ± 0.54	1.60 ± 1.50	0.030

SD: standard deviation
*Wilcoxon signed-rank test

Table 2. Comparison of post-surgery clinical parameters between test and control groups

Clinical parameter	Post-surgery (mm) (mean ± SD)		P*
	Test	Control	
Probing pocket depth	1.00 ± 0	1.00 ± 0	-
Width of keratinized gingiva	5.00 ± 0.63	2.60 ± 1.50	0.030
Width of attached gingiva	4.30 ± 0.51	1.60 ± 1.50	0.010

SD: standard deviation
*Wilcoxon signed-rank test

Also, the post-surgery clinical parameters of test and control groups are compared and shown in table 2. According to these results, mean of the increased width of keratinized and attached gingiva was 4.17 mm and 4.14 mm in test and 1.10 mm and 1.10 mm in control sites, respectively, 3 months after surgery. The difference between the width of keratinized gingiva in test and control sites was significant (P = 0.030). Likewise, the comparison of attached gingiva width between control and test site was also statistically significant (P = 0.010) (Table 2). The comparison between the width of keratinized and attached gingiva before and after surgery showed a significant difference in both test and control groups (P = 0.001, and P = 0.030 respectively) (Table 1).

Discussion

In dentistry, the culture of epithelial cells for regeneration of oral soft tissue was performed, at the first.[13,14] During the healing, the epithelial sheets are weak against mechanical trauma, and manipulation of them during surgery is difficult.[14] Because of these disadvantages, culture of fibroblasts for preparation of tissue-engineered soft tissue grafts was indicated.[15,16,19-21] According to the study carried out by Karring et al., the morphogenetic stimuli of the underlying connective tissue control the keratinization of gingival epithelium.[27] Therefore, culture of the fibroblasts was performed to produce tissue-engineered soft tissue grafts in several studies.[15,19,20,22] In this study, we used fibroblasts and an autologous scaffold (PRGF) to augment the gingiva. Both control and test sites showed a significant increase of keratinized and attached gingiva width, 3 months after surgery. The average increase

in keratinized and attached gingiva was 4.17 mm and 4.14 mm, respectively. At control sites, the average increased width of keratinized and attached gingiva was 1.10 mm which was statistically significant. The difference between the control and test groups in regard to the width of keratinized and attached gingiva was 3.06 mm and 3.04 mm, respectively. In a study done by Prato et al., benzyl ester of hyaluronic acid was introduced as a scaffold.[15]

Gingival fibroblasts were cultured and seeded onto this scaffold and this prepared tissue was grafted onto a periosteal bed. The average increased keratinized tissue was 2.00 ± 0.41 mm. In this study, the keratinized gingiva increased about 2.17 mm more than aforementioned study. The difference between the type of scaffold and bed preparation (marginal incision in Prato et al. study and sub-marginal incision in our study) can explain this result. Another study evaluated a living human fibroblast-derived dermal substitute (HF-DDS) and compared it to a gingival autograph (GA).[19] McGuire and Nunn used an absorbable polyglactin scaffold and allogeneic dermal fibroblasts. The average of increased keratinized gingiva around the teeth with inadequate attached gingiva was 2.72 mm after 3 months post-surgery. In this study, although the amount of increased keratinized tissue was less in test sites, the esthetic result was better than control sites.

Mohammadi et al. in a study, used a collagen type I (Zyderm) as a scaffold which human gingival fibroblasts were seeded into it.[20] Results showed mean increased width of keratinized and attached gingiva to be 2.8 mm in test (TEGG) and 1.9 and 2 mm in control sites (periosteal fenestration

technique), respectively, 3 months after surgery. When test and control groups were compared, the width of keratinized and attached gingiva clinically increased (0.9 mm and 0.8 mm, respectively). In the present study, about 1.37 mm keratinized gingiva and 1.34 mm attached gingiva was obtained more than the results of Mohammadi et al.[21] study. In these two studies, bed preparation (periosteal fenestration), dimensions of recipient bed, incisions, cells (fibroblast) were similar. Only, the difference between the scaffolds can infer these results. Mohammadi et al. in a case report, showed that using a cultured gingival graft (fibroblasts + collagen scaffold) can increase the width of pri-implant keratinized tissue.[21]

In an animal study, chitosan was used as a carrier for transporting fibroblasts to the recipient bed prepared in the mouth of dogs, in order to gingival augmentation.[22] At test sites (chitosan + fibroblasts), the width of keratinized gingiva showed a 2.13 mm increase in mid-buccal surface of teeth. At control sites (chitosan), the width of keratinized gingiva increased about 1 mm. The difference between test and control sites was not statistically significant. Scheyer et al. showed that application of living cellular sheet in the treatment of mucogingival defects resulted in better color, absence of scar, and better mucogingival junction alignment in comparison with free gingival graft.[28]

The present study showed the most increased keratinized and attached gingiva width among the studies performed in the field of soft tissue engineering. The reason which can explain this difference is the type of scaffold used in this study (PRGF). As we know, PRGF is a combination of fibrin and platelets growth factors. Platelet growth factors existed in α-granules become activated after preparation of PRGF. Growth factors can affect angiogenesis, proliferation of fibroblasts, mutagenesis, extracellular matrix (fibronectin, glycosaminoglycan) and collagen synthesis which have key roles in regeneration of soft tissue.[27]

The use of tissue-engineered gingival grafts has several advantages including requiring a small donor site, obtaining enough keratinized tissue, less complaint and discomfort emerges for patients, and being safe and less costly because an autologous scaffold is used. According to this study, these tissue-engineered materials effectively create keratinized gingiva. Of course, further controlled clinical trial is needed.

Conclusion

Results of this investigation show that the tissue-engineered gingival graft is able to generate keratinized tissue safely and with little complication. Our suggestions are using PRGF alone at control sites in the next studies, and increasing the sample size for enhancing the power of the study.

Conflict of Interests

Authors have no conflict of interest.

Acknowledgments

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