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ORIGINAL ARTICLE

# Vascularization after treatment of gingival recession defects with platelet-rich fibrin or connective tissue graft

Gülnihal Eren[1] · Alpdoğan Kantarcı[2] · Anton Sculean[3] · Gül Atilla[1]

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**Abstract**
*Objective* The aim of this study was to evaluate histologically the following treatment of bilateral localized gingival recessions with coronally advanced flap (CAF) combined with platelet-rich fibrin (PRF) or subepithelial connective tissue graft (SCTG).
*Materials and methods* Tissue samples were harvested from 14 subjects either 1 or 6 months after the surgeries. The 2-mm punch biopsies were obtained from the mid-portion of the grafted sites. Neutral buffered formalin fixed, paraffin-embedded 5-μm thick tissue sections were stained with hematoxylin eosin and Masson's trichrome in order to analyze the collagen framework, epithelium thickness and rete-peg length. Multiple sequential sections were cut from paraffin-embedded blocks of tissue and immunohistochemically prepared for detection of vascular endothelial growth factor, CD31 and CD34, for the assessment of vascularization.
*Results* Rete peg formation was significantly increased in the sites treated with PRF compared to the SCTG group after 6 months ($p<0.05$). On the contrary, the number of vessels was increased in the SCTG group compared to the PRF group after 6 months ($p<0.05$). No statistically significant differences were observed in the collagen density. Staining intensity of CD31 increased in submucosal area of PRF group than SCTG group after 1 month. Higher staining intensity of CD34 was observed in the submucosal area of PRF group compared with SCTG group after 6 months.
*Conclusions* The results of the present study suggest that in histological evaluation because of its biological compounds, PRF results earlier vessel formation and tissue maturation compared to connective tissue graft.
*Clinical relevance* PRF regulated the vascular response associated with an earlier wound healing.

**Keywords** Subepithelial connective tissue graft · Gingival recession · Platelet-rich fibrin · Histology · Wound healing

✉ Gülnihal Eren
gulnihal_karasu@hotmail.com

[1] School of Dentistry, Department of Periodontology, Ege University, Bornova-İzmir, Turkey

[2] Department of Applied Oral Sciences, Center for Periodontology, The Forsyth Institute, Cambridge, MA 02142, USA

[3] Department of Periodontology, School of Dental Medicine, University of Bern, Bern, Switzerland

## Introduction

One of the main goals of periodontal plastic surgery is the full and predictable coverage of exposed root surfaces [1]. Recession defects are associated with dentinal hypersensitivity, root caries, impaired plaque control, and esthetic complications [2]. Buccal gingival recessions in the anterior region represent one of the most challenging soft tissue problems. Various methods, such as free gingival graft, laterally positioned flap, coronally advanced flap (CAF), guided tissue regeneration with membranes, subepithelial connective tissue graft (SCTG), acellular dermal matrix, enamel matrix derivative, platelet-rich plasma, platelet-rich fibrin (PRF), or combination techniques with CAF are available for treatment of gingival recession defects [3–5]. SCTG shows the greatest predictability and is accepted as the gold standard [6–9]. While the goal of root coverage is achieved, many of these procedures including the SCTG may not be fully sufficient in establishing homeostasis of the mucogingival complex and regenerating the lost attachment apparatus including the formation of the new cementum with inserting connective tissue fibers and regeneration of alveolar bone [10].

Various animal studies and case reports have examined the histologic interface between the root surface and the grafted tissue after root coverage procedures [2, 11–16] suggesting that CAF and SCTG were associated to some degree of periodontal

regeneration [11–13]. Healing predominantly occurs by the formation of a long junctional epithelium or a connective tissue adhesion interface between the root surface and the graft [2, 14]. In spite of predictable clinical outcomes with the use of CAF + SCTG, the mechanism of healing process still remains controversial, due to the lack of human histological evidence. A single case report [17] has demonstrated that the histologic soft tissue healing of SCTG with CAF grafted tissue from humans was characterized by dense fiber formation. This phenomenon could provide long-term stability.

PRF is an autologous fibrin matrix and classified as a second-generation platelet concentrate because it does not require anticoagulant while preparation, and it contains 50 % of leukocytes of the original blood volume [18–21]. Previous in vitro studies showed a slow release of growth factors, such as transforming growth factor-β1, platelet-derived growth factor, and vascular endothelial growth factor from PRF during the first 7 days into a sterile medium [22, 23]. Even though SCTG and PRF involve different strategies, comparable procedures in the treatment of localized gingival recessions [24, 25] have suggested that they could be associated with similar clinical outcomes. Yet, the mechanism of the wound healing is still unknown. We have hypothesized that the early wound healing of PRF after treatment of localized gingival recessions is driven by a vascular response, which regulates the tissue regeneration. Therefore, the aim of this paper was to histologically study and compare the healing and revascularization of the soft tissues after treatment of localized gingival recessions with CAF + PRF and CAF + SCTG at different time points.

## Materials and methods

### Patient selection and experimental design

Fourteen subjects ranging from 18 to 45 years of age, with similar bilateral or contralateral Miller Class I or II [26] localized gingival recessions of at least ≥2 mm, located on incisors, canines, or premolars on both jaws and gingival thickness at least ≥0.8 mm for the recession area were recruited. All patients participated on a voluntary basis and signed a consent form after the procedures were clearly explained. The tissue samples were obtained in agreement with a protocol approved by the Ethics Committee of the Ege University Izmir, Turkey (2012; no:12–12.1/19).

Test group of patients received PRF + CAF treatment while the control group received CTG + CAF [24]. Before surgery, intravenous blood was collected in a 10-mL glass-coated plastic tube without any anticoagulant and centrifuged immediately using a table centrifuge (Nüve Laboratory Equipments, NF200, Ankara, Turkey) for 12 min at 400 ~ g as suggested by Dohan et al.[18]. The preparation of the recipient bed and suturing technique were identical in both groups. Sulcular incisions were made on the recipient teeth and joined to horizontal incisions extending into the adjacent interdental areas slightly coronal to the CEJ. A trapezoidal-shaped, partial-thickness flap was elevated, providing a vascular connective tissue bed for placement of the selected graft material. At each site, either a PRF membrane (2-mm thick) or a CTG (1.5-mm thick) placed over the defect. Both grafts were secured to the interdental papillae and adjacent soft tissue at the apical part with horizontal mattress sutures. Each partial-thickness flap was further released and positioned over the graft to cover the CEJ and sutured with 7–0 polypropylene sutures.

### Tissue specimens

Gingival tissue samples were harvested at 1 or 6 months after the root coverage procedure. One-month biopsies were harvested from 9 patients, and 6-month biopsies were harvested from 5 patients using a 2-mm punch biopsy pen under local anesthesia. Incision was performed positioning the handheld disposable punch perpendicular to the tooth and 2 mm away from the gingival margin, performing simultaneous rotational movements under gentle pressure. Biopsy was excised from the mid-portion of the healed recession area. A needle was inserted into each specimen mesio-distally in order to obtain orientation (Fig. 1).

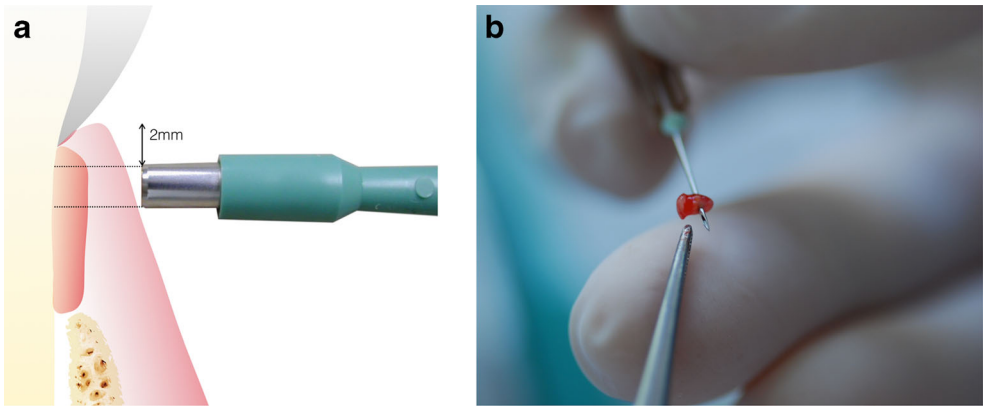


**Fig. 1** **a** At 1 month or at 6 months, a punch biopsy needle was inserted 2 mm away from the gingival margin. The instrument was rotated down through the gingiva until the touch of bone, producing a cylindrical core of tissue. **b** To ensure the orientation is held constant for imaging and slicing, a needle has inserted through the mid portion of the specimen parallel to the epithelium

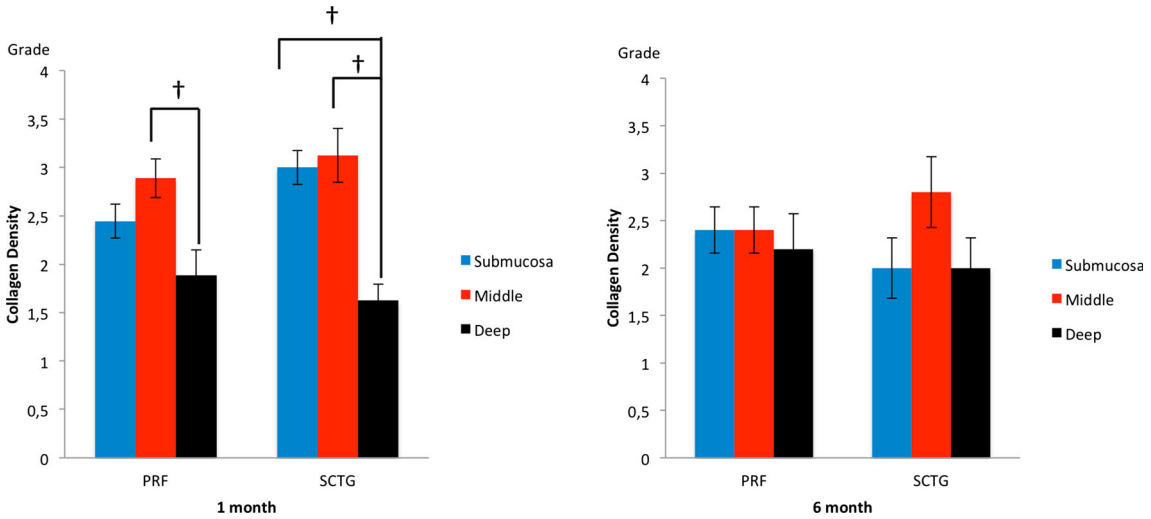


**Fig. 2** Collagen density in different sites of the tissues after PRF and SCTG treatment. ($^{\dagger}p < 0.05$ within the same group)

## Histology and immunohistochemistry

All specimens were fixed in 10 % neutral buffered formalin solution for further descriptive histological analyses. Following dehydration, specimens were embedded in paraffin at buccal-lingual direction; oriented for sectioning as perpendicularly to the surface plane as possible. Five-micrometer-thick sections were collected serially and thaw-mounted on glass slides. All sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (M&T) stain for observation of the collagen framework.

Immunofluorescence staining was performed to study VEGF, CD31, and CD34 using mouse monoclonal anti CD31 antibody (1:25; Santa Cruz Biotechnology, Santa Cruz, CA; catalogue number sc-19587), mouse monoclonal anti CD34 antibody (1:25; Invitrogen, Camarillo, CA; catalogue number 37–0700) or with polyclonal VEGF antibody (1:25; Santa Cruz Biotechnology, Santa Cruz, CA; catalogue number

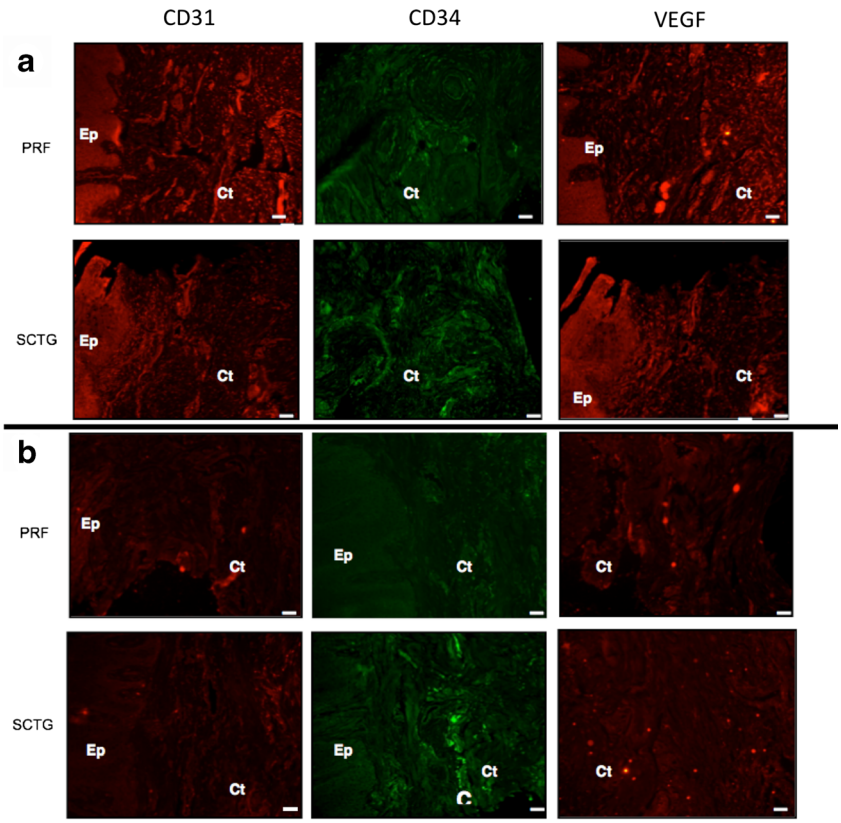


**Fig. 3** Immunofluorescence staining of PRF and SCTG treated tissue samples at 1 month (**a**) and at 6 months (**b**). Bar = 50 μm

sc-152). Sections were treated with fluorescent secondary antibodies (Alexa Fluor-mouse 635 for CD31 and Alexa Fluor-rabbit 568 for VEGF) and incubated in the dark for 1 h. This step was skipped for mouse monoclonal anti CD34 antibody. Tissue sections were visualized under a fluorescence microscope (Zeiss Axio Observer, Jena, Germany).

The images were captured by a digital camera at an original magnification of ×10, ×20, and ×40. Axio Observer software was used for the image analysis. The collagen density was measured by the distribution of collagen fibers in the total inspection area as follows: grade 1: loose; grade 2: moderate; grade 3: dense; grade 4: very dense [27]. The degree of the CD31, CD34 and VEGF was characterized by fluorescence immunostaining methods as described [28].

### Statistical analysis

The mean value for each parameter was obtained per tooth. The mean values for both groups were determined by using the individual means from subjects. After confirming a normal distribution, the paired *t* test was used to check the hypothesis of no difference between the two groups with regard to the evaluated parameters. $P < 0.05$ was considered statistically significant.

## Results

Both groups showed less dense collagen fibers in deep sites of the tissues at 1 month. In the PRF group, density of the collagen fibers was higher in the middle site than the deep site at 1 month ($p = 0.02$). SCTG group showed less dense collagen fibers in deep sites compared to the middle and submucosa sites at 1 month ($p < 0.001$, $p < 0.0001$; respectively). The density of the collagen fibers was uniform in all sites of the tissues in both groups at 6 months (Fig. 2).

Increased neovascularization was found in the connective tissue in various sizes for both groups. The location of the

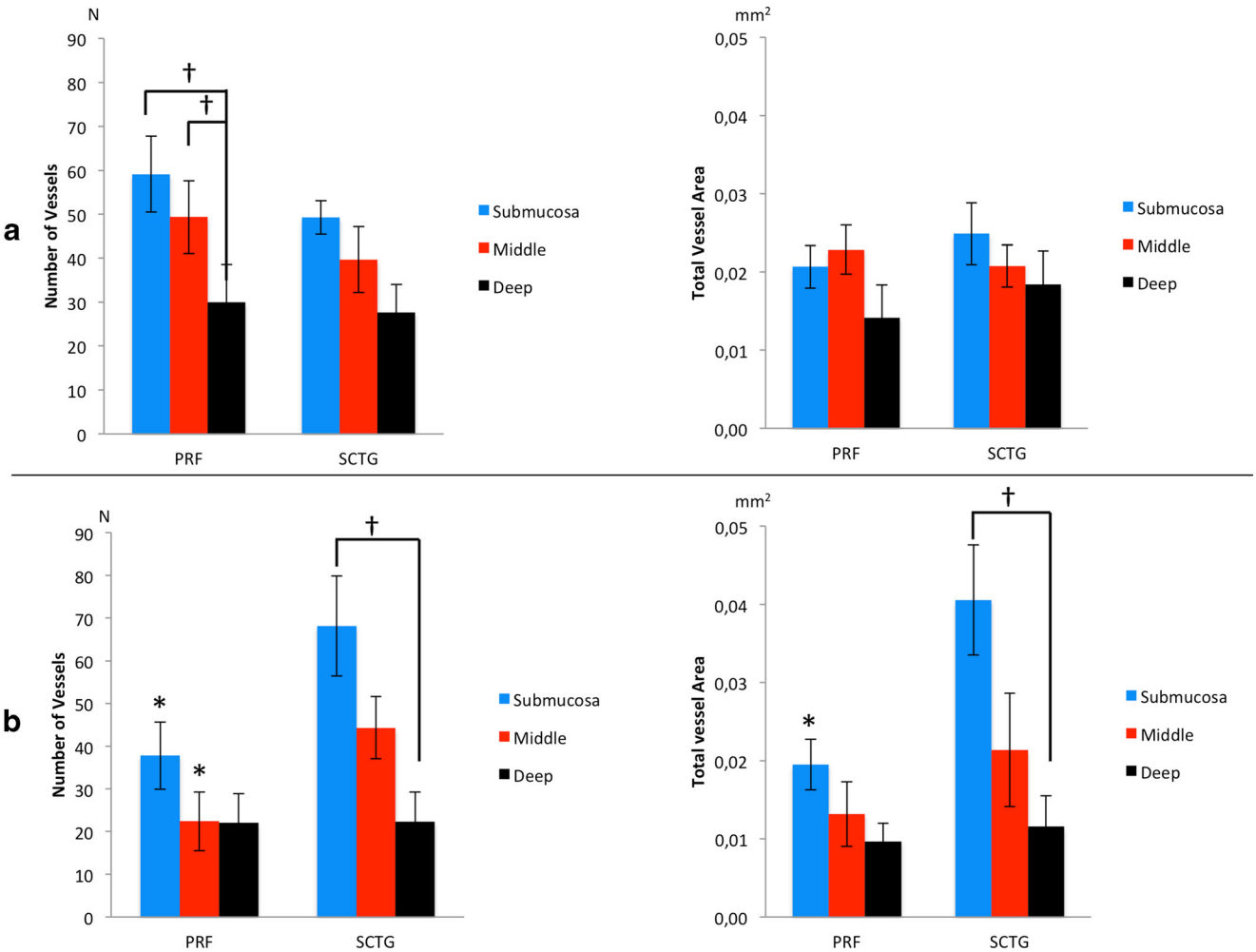


**Fig. 4** Vascularization characteristics in different sites of the tissues at 1 month (**a**) and at 6 months (**b**). (*$p < 0.05$ between the groups, †$p < 0.05$ within the same group

vessels positive to these molecules immunostaining demonstrated that proliferation and growth of new vessels were uniformly distributed within the connective tissue of both PRF and SCTG treated groups (Fig. 3). CD31, CD34, and VEGF expression were significantly higher in the SCTG group at 6 months, suggesting an ongoing vascularization.

PRF group presented less number of vessels in deeper sites compared to middle and submucosa layers at 1 month ($p = 0.05$, $p = 0.005$; respectively). SCTG group showed statistically higher numbers of vessels than PRF group in submucosa and middle portions of the tissues ($p = 0.025$, $p = 0.024$; respectively) at 6 months (Fig. 4). The number of vessels in submucosa layers was higher than that of the deep sites in SCTG group at 6 months, and the difference was significant ($p = 0.003$).

All treated sites were characterized by connective tissues covered with keratinized epithelium at 1 month after grafting (Fig. 5). Epithelium showed elongated rete-pegs. Newly formed vessels and collagen deposition were observed. The two groups presented similar epithelium thickness, epithelium area, and rete-peg length at 1 month ($p > 0.05$). Histological analysis of the 6-month gingival samples showed mature and uniform collagen deposition. SCTG specimens showed evidence of newly formed large vessels (Fig. 6). Rete-peg length was reduced in the SCTG group when compared to the PRF group ($p = 0.05$) at 6 months. Number of vessels and total vessel area were higher in the SCTG group than the PRF group at 6 months ($p = 0.035$, $p = 0.028$; respectively).

## Discussion

The ultimate goal of periodontal plastic surgery is the complete root coverage. Although the SCTG still holds the most promise in terms of root coverage, only the histological examination can reveal cellular events unrolled into the grafted tissue-root surface interface. With the presence of growth factors and matrix glycoproteins, PRF supports cell migration, wound healing, and tissue regeneration [29]. Although the clinical use of PRF has been widely adopted in the treatment of gingival recessions [24, 25, 30, 31], histological data has been available for only a few clinical studies. The aim of this study was to histometrically evaluate the healing process of gingival recessions treated with PRF in combination with CAF and to compare it to that obtained using SCTG in combination with CAF.

In a recent study, we evaluated the efficacy of PRF in the treatment of gingival recessions [24]. The findings of that

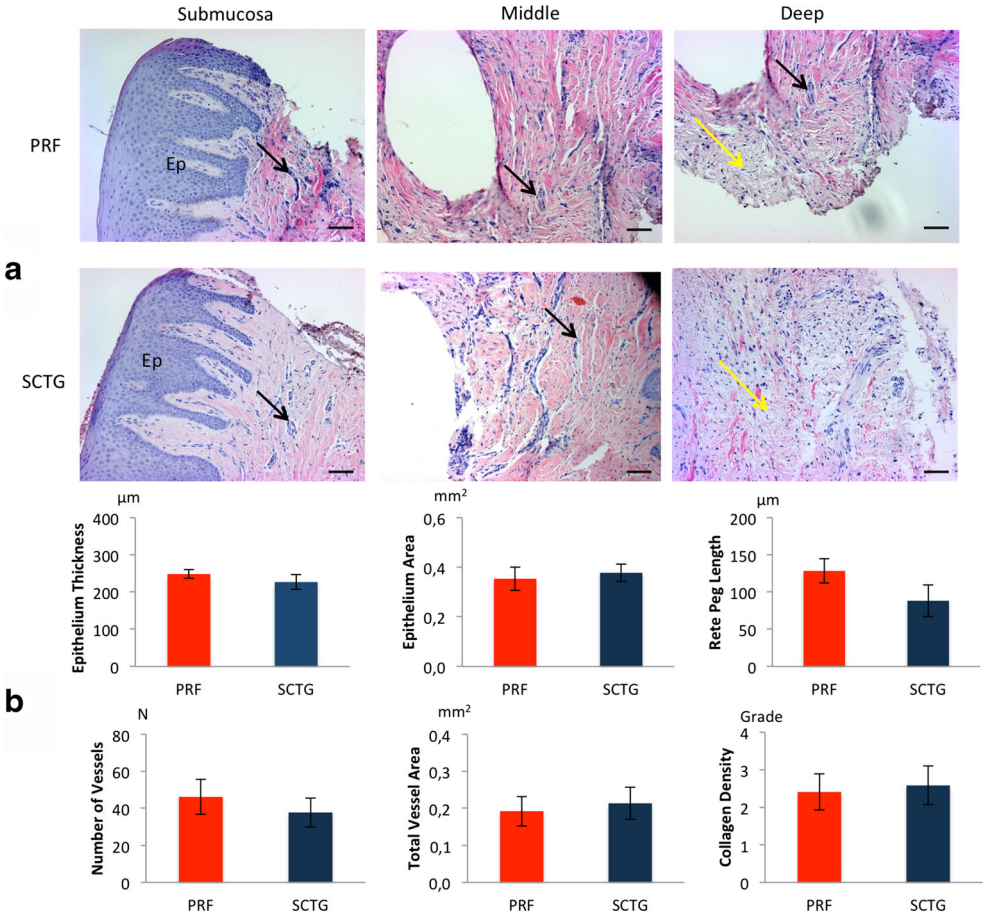


**Fig. 5** **a** PRF and SCTG treated sites at 1 month (hematoxylin eosin; original magnification ×100, bar = 50 µm). **b** Characteristics of the epithelium, vascularization, and collagen density after treatment with PRF or SCTG at 1 month (*$p < 0.05$ between the groups)

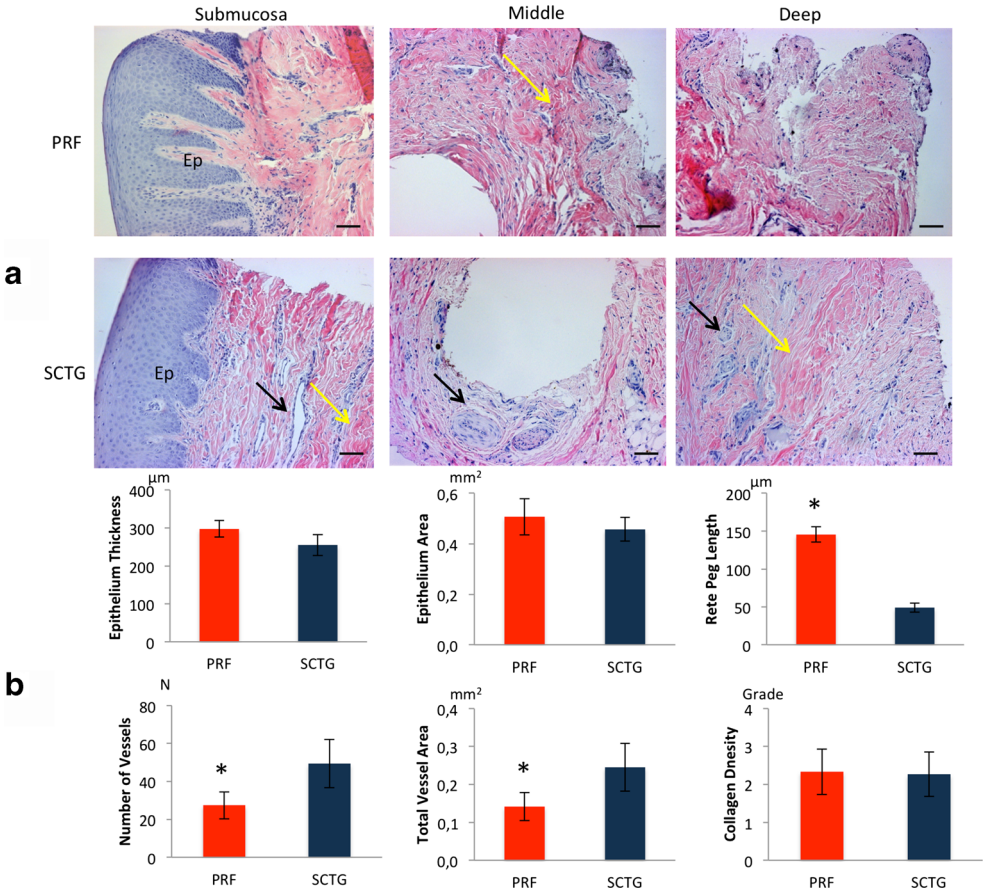


**Fig. 6** **a** PRF and SCTG treated sites at 6 months (hematoxylin eosin; original magnification ×100, bar = 50 µm). **b** Characteristics of the epithelium, vascularization, and collagen density after treatment with PRF or SCTG at 6 months (*$p < 0.05$ between the groups)

study demonstrated that localized gingival recessions could be successfully treated with CAF + PRF as well as CAF + SCTG, and PRF might be suggested as an alternative to SCTG for the treatment of localized gingival recessions. In the present study, the healed tissues of recession areas treated with CAF + PRF or CAF + SCTG were analyzed histologically; the connective tissue by Masson's trichrome to demonstrate the density of collagen fibers, the epithelium, and vascularization characteristics by H&E stain. Also, CD31, CD34, and VEGF molecules were documented. The histologic findings revealed good integration in the epithelial layer of the tissues with the recipient sites in both groups. Guiha et al. [32] showed that at 60 days after CAF + SCTG treatment, the epithelium regained its normal shape, thickness, and appearance. The thickness of the epithelium of CAF + PRF treated areas was similar to CAF + SCTG, except the rete-peg length. PRF group showed much deeper rete-pegs than the SCTG group at 6 months biopsies. These well-developed rete-pegs in the keratinized epithelial layer may provide mechanical resistance to external irritation.

The quantity of vessels, fibroblast, and collagen fibers; the manner in which these structures position themselves within the tissue; and the thickness of the epithelium and rete-peg layer are all factors that determine the maturity of the gingival tissue formed after grafting [33]. Guiha et al. [32] studied the healing and revascularization of the SCTG. They have reported that graft is vascularized at 7 days after surgery. By the end of the second week, vascularization decreased and at 28 days, the microvasculature of the flap and the graft was almost normal. In the present study, both PRF and SCTG groups showed similar vascularization patterns in number of vessels and vessel area at 1 month.

VEGF represents a specific mitogen of endothelial cells in vitro and an inducer of angiogenesis in vivo [34]. VEGF expression is significantly increased in the healing stage of periodontal disease [35]. CD31 is a cell adhesion molecule, which plays a key role in adhesion between endothelial cells and in interactions with leukocytes [36]. On the other hand, CD34 is highly expressed on the surface of regenerating endothelial cells and is a marker of proliferating endothelial cells in the growing sprouts during angiogenesis [37, 38]. Gingival samples were stained positively for CD31 in the inner ring of the vessels, and CD34 was intensely expressed by endothelial cells [39]. Our findings were in accordance with the previous studies [35, 37, 39] suggesting that CD31 and CD34 might have different roles during gingival inflammation.

In the present study VEGF, CD31 and CD34 primary antibody staining showed increased vessel formation at 1 month in both groups. Additionally, increased small-diameter vessels evaluated microangiographically support

the histologic findings. It has been shown that, maturation of the vasculature had been achieved at 60 days after treatment of gingival recessions with CAF + SCTG [32]. However, in the present study at 6 months, the number of vessels and total vessel area were higher in the SCTG group than the PRF group. There may be several explanations for this finding. First, this increased angiogenesis at 6 months possibly accounts for increased graft viability. As shown in a recent study [40], incorporation of endothelial cells promoting the formation of a well-organized capillary network can enhance tissue-engineered skin vascularization. Second, on the contrary, it might be related to slow healing of SCTG treated recessions, and high vascularization might show ongoing maturation of the tissue. We thus suggest that PRF might enhance angiogenesis and neovascularization through the release of pro-angiogenetic factors in the early phases of wound healing, therefore showing faster healing than SCTG at 6 months. This view is supported by an animal study by Sun et al. [41]. They reported that in acute myocardial infarction, animals treated with mesenchymal stem cells PRF scaffold showed of augmented angiogenesis activities, including the upregulated expressions of angiogenesis factors at 42 days than those treated by direct mesenchymal stem cells implantation [41].

The collagen matrix determines differentiation and cell proliferation [42]. Proteins present in the matrix, such as collagen, elastin, fibronectin, and proteoglycans among others, promote phenotypic changes in undifferentiated mesenchymal cells, and the tridimensional structure of the matrix allows cell adhesion, which is essential for fibroblasts to initiate mitosis or the production of extracellular matrix. In the present study, the density of the collagen fibers was uniform in all parts of the tissues in both groups at 1 and 6 months. The integrity of the collagen structure is as important as the presence of viable cells for the success of the treatment [43]. The present results suggest that PRF and SCTG sites showed similar appearance of connective tissue composition.

CTG technique is accepted as the gold standard for obtaining complete root coverage and shows a great degree of predictability [44]. On the other hand, CTG requires a second surgical donor site in the palate and may lead to patient discomfort [45]. The use of PRF has been tested as an alternative to the CTG in the treatment of localized gingival recessions in order to eliminate the need for a second surgical procedure in the palate [24, 25]. These studies demonstrated that the clinical efficacy of the PRF was comparable to the CTG over a period of 6 months. In the present study, we have further hypothesized that the PRF would not only result in a similar clinical outcome but will also enhance the wound healing, which would be critical for the vascularization of the grafted area and predictability of the long-term success. Our work revealed that PRF regulated the vascular response associated with an earlier wound healing.

## Conclusion

Studying the characteristics of the tissue such as vascularization, collagen density, and epithelium thickness is subjective but also valid for evaluation parameters of wound healing and angiogenesis activities. The results of our study demonstrated that in histological evaluation CAF + PRF results earlier vessel formation and tissue maturation compared to connective tissue graft.

### Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no competing interests.

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**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of Ethics Committee of the Ege University Izmir, Turkey (2012; no:12–12.1/19) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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