Original Article

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Primary contraction of harvested palatal sub-epithelial connective tissue grafts: a pilot study

Chanutda Chatchaiyadej[1], **Wichurat Sakulpaptong**[2]

[1] *Residency Training Program in Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand*
[2] *Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand*

Objective(s): To elucidate and compare the primary contraction of palatal sub-epithelial connective tissue grafts after harvesting at different times.
Materials and Methods: Ten patients who underwent soft tissue graft treatment, free gingival graft, or connective tissue graft. A 4-mm biopsy punch took each sample with 1 mm thickness after 1-mm-depth de-epithelialization. A total of 10 samples were recorded for graft contraction by a standardized photograph taken at 20(T1), 40(T2), 60(T3), 90(T4), 120 minutes(T5), and 24 hours(T6). Throughout the contraction investigation, the sample was incubated at 37 degrees Celsius, washed twice, and immersed in a new normal saline solution at 4 degrees Celsius between time points. The graft area was computed using ImageJ software. The graft contraction was calculated as the average percent of the original area (% of T1). Differences in graft contraction were analyzed using Friedman's Two-Way Analysis of Variance by Ranks and pairwise comparisons.
Results: Only one significant difference in graft contraction was found between T4 and T2, meaning that the graft area at 90 minutes decreased compared to the graft area at 40 minutes (*p-value =0.047*).
Conclusion: The harvested palatal sub-epithelial connective tissue graft size remained constant, except at 90 minutes after harvesting when it significantly decreased compared to 40 minutes. This study indicates that the sub-epithelial connective tissue graft can stay in normal saline solution outside the oral cavity with minimal contraction.

Keywords: connective tissue graft, contraction, harvesting time points

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Introduction

The gingiva consists of free and attached gingiva, with keratinized gingiva serving as an integral component of this structure [1]. The width of the keratinized gingiva is of paramount importance in maintaining oral hygiene, particularly in teeth with subgingival restoration [2]. For a dental implant, an adequate zone of attached gingiva is crucial for ensuring good oral hygiene, pocket depth, and aesthetic considerations [3, 4].

Mucogingival deformities definition varies, they can be congenital, developmental, or acquired defects. These deformities can occur on natural teeth sites, implant sites, or edentulous areas. Moreover, mucogingival deformities are not limited to soft tissue sites; but they can also occur in hard tissue area, causing bony deformities [5]. In general, mucogingival deformities refer to conditions that deviate from normal range of physiological variations [6].

Mucogingival deformities and other periodontal conditions surrounding teeth need to be considered. These deformities are considered an important problem in periodontal disease and other conditions affecting the periodontium part, according to the 2017 World Workshop in the classification of periodontal and peri-implant disease and condition. This consensus is also supported by the American

Corresponding author: Wichurat Sakulpaptong
Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University
6 Yothi Street Ratchathewi, Bangkok 10400 Thailand
Tel: +66 220O 7841 Fax: +66 2200 7840 E-mail: wichurat.sak@gmail.com
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Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) [7].

If patients maintain good oral hygiene despite the presence of mucogingival deformities and other unfavorable conditions, they are more likely to maintain periodontal health. However, these conditions may differ if patients require orthodontic, implant, and restorative treatments [6]. Gingival/soft tissue recession, identified as a mucogingival deformities, is one of the common problems in patients. Gingival recession (RE) is described as the apical displacement of the gingival margin in comparison to a cementoenamel junction (CEJ) [5].

RE increases with age [7]. RE was found to have a prevalence of 49.6% among 51- to 59-year-old individuals and a higher rate of 72.0% in individuals above 70 years old. The study reported that males had a greater level of gingival recession than females. Moreover, the percentage of RE at the buccal site was associated with age, gender, calculus, and cervical abrasion, while the percentage of RE in all sites was not associated with cervical abrasion. This suggests that different RE sites may involve various processes, and there is a need for further research on site-specific factors [8].

RE can be either generalized or localized. Several predisposing factors or etiologies of the RE have been proposed, such as occlusal trauma, incorrect brushing technique, and the presence of mucogingival defects, including thin gingival biotype, high frenum attachment, and lack of keratinized gingiva [9].

Moreover, RE has a correlation with those who have a history of orthodontic treatment, especially in young patients [10]. Patients whose gingiva has recession may also experience tooth sensitivity, a high risk of root caries, and compromised esthetics [9]. These problems would increase patients' concerns and eventually lead them to seek help from dentists. In some cases, patients may require some further dental restorations, fillings or prostheses, or root coverage treatment to treat the RE and prevent tooth loss [6].

The coronally advanced flap with connective tissue graft (CTG) is considered the gold standard for augmenting soft tissue volume at implant sites, partially edentulous sites for fixed prosthesis, and for treating RE by root coverage [11]. When there is insufficient soft tissue around teeth or implants, soft tissue augmentation is needed to improve its quantity and quality. A CTG is considered one of the best treatment options to improve soft tissue volume and increase the width of keratinized tissue [5]. Another advantage of CTG treatment is correcting RE through a procedure called root coverage [7]. Furthermore, it is a predictable treatment with stable outcomes, making it a reliable option for patients [2, 4, 12].

The most common site generally used for harvesting CTG is the palate [12, 13]. This CTG harvested from the patient's palate offers several advantages, including minimal graft rejection, acceptable esthetic outcomes, and reduced material costs [14]. Many techniques for harvesting the CTG are proposed [13]. The techniques are usually divided into two options. One option involves the donor site being left with a raw surface, which is known as de-epithelialization of FGG. The other option involves little to no raw surface at the donor site, such as a single or double incision with a partially raised flap and CTG taken from below the flap [15].

Compared with the de-epithelialization of FGG, in which the donor site became a raw surface, the CTG taking techniques with preservation of the flap cause less pain to the patient as they result in smaller open wounds after the graft is taken [16]. According to the literature, the process of harvesting CTG can be adapted to minimize the duration of grafts outside the oral cavity, thereby reducing the likelihood of graft contraction or shrinkage [13, 17, 18].

The graft contraction is divided into primary and secondary contraction. The primary contraction starts when the graft separates from the donor tissue and is placed into the recipient site. The secondary contraction is from the graft attached to the recipient to the completed healing. The thickness of the grafts also impacts the rate of contraction. The graft: a thick graft tends to undergo more primary contraction, whereas a thin graft tends to undergo more secondary contraction [18].

During treatments, the graft is commonly taken from the patient's palate [2, 18, 19]. Its thickness and length are determined by anatomical features such as the greater palatine foramen and vessels, palatal rugae, and the height of the palate [20-23].

The size of the graft is tailored to fit the requirements of the treatment area. However, a common concern is shrinkage of the graft area due to graft contraction. Previous studies have reported that CTG treatment may result in secondary contractions, ranging from 28% to 37% within about six months, which further increased to 43% within a year [15, 24]. Despite the existing studies on secondary graft contraction, research on primary graft contraction is still limited.

In periodontal surgery treatment, the success rate is affected by various factors. One such factor is the blood supply to the graft placed over the denuded root, where there is a lack of blood supply [17]. The blood supply comes from the margin of the recipient bed and some capillaries in the graft [18]. Adequate blood supply is imperative for the survival of the graft. Inadequacy of blood supply can result in partial necrosis, which can adversely affect the graft's vitality. Furthermore, if the graft contracts to the point where there isn't enough graft area to receive nutrients from the recipient bed and support the graft on the exposed root area, this could result in reduced graft survival and less effective root coverage.

Consequently, due to limited information on the CTG contraction after harvesting, this research aims to elucidate the primary graft contraction of palatal sub-epithelial CTG after harvesting and submerging them in normal saline for 20, 40, 60, 90, 120 minutes, and 24 hours

Objectives

The aim of the present study was to investigate and compare the primary contraction of palatal sub-epithelial CTG after immersing them in sterilized normal saline solution for 40, 60, 90, 120 minutes, and 24 hours.

Materials and Methods

The study was conducted on 10 adults, who underwent periodontal soft tissue graft treatment, specifically free gingival graft (FGG) and connective tissue graft (CTG), at the Periodontology clinics within the Faculty of Dentistry at Mahidol University in Thailand.

The inclusion criteria provided as follows: eligible individuals must be at least 18 years of age, exhibit controlled systemic conditions or be systemically healthy, require periodontal soft tissue graft treatment for palatal donor harvesting, and maintain appropriate levels of inflammation control (with a full-mouth bleeding on probing ≤ 25%) and plaque control (with a full-mouth plaque score ≤ 25%)

To ensure safe and effective surgical procedures, exclusion criteria must be established. Patients who have smoking habits, inflamed gingival tissue, presence of pathologic oral lesions, inadequate palatal mucosa after donor harvesting, or who have taken antibiotics within six months prior to surgery are not eligible. Patients must also provide signed informed consent to participate in the research and have extra-site surgery before proceeding

with the surgical procedure, both normal treatment surgery by the resident provider and harvesting samples by the researcher.

The study protocol was approved by The Ethics Committee for Human Subjects at the Institutional Review Board, Faculty of Dentistry and Faculty of Pharmacy (MU-DT/PY-IRB) on 8 Mar 2023 (No. COA MU-DT/PY-IRB 2023/024.0803). All participants provided informed consent before the study commenced. The study was conducted following the principles of the Helsinki Declaration on Human Experimentation, which was adopted in 1975 and revised in 2008/2013. Recruitment occurred between May and November 2023. The protocol involved through communication with the patient, resident provider, and instructor regarding the procedure, potential risks, and risk management.

After being given permission from the volunteers, the researcher collected data on age, gender, race, weight, height, medical conditions, medications, and history of periodontal disease and treatments.

For each subject in the study, a 4-mm biopsy sample with a thickness of 2 mm was taken from an area located 2 mm adjacent to the typical palatal soft tissue graft harvest site, using a 4-mm biopsy punch. Intraorally, each biopsy sample was de-epithelialized to a depth of 1 mm by a blade before being removed from the donor bed. This de-epithelialized biopsy sample with a 1-mm thickness represents a CTG for this study. This study chose the de-epithelialized technique for obtaining CTG because of limited donor site area. This technique is easy to perform and requires a short procedure time. Although this technique caused raw wound surface, the biopsy was only 4-mm diameter and was located near the treatment surgery site. Therefore, the patient did not experience more additional pain than the routine surgical treatment that the patient received. Each patient was scheduled for routine follow-up appointments at one week, two weeks, one month, and three months.

After CTG detaching from the bed, the graft sample was immediately immersed in sterile normal saline solution (NSS) for five seconds to remove excess blood, then placed in a 24-well plate containing 10% PrestoBlue® solution in NSS (Invitrogen, USA). The Prestoblue® viability data is unpublished. The time of sample removal from the bed was recorded as the starting point for the graft being outside the oral cavity. At each time point, 20 (T1), 40 (T2), 60 (T3), 90 (T4), 120 (T5) minutes, and 24 hours (T6), after the sample was incubated at 37°C, the sample was rinsed with NSS twice to remove excess Prestoblue®. Then, it was photographed at the photo station, the distance of which had already been predetermined. The photos were captured using a Samsung Galaxy S22+ mobile camera in Pro mode with a 3 times magnifier, taking 3 photos for each front and back view, resulting in a total of 6 photos. A ruler was placed over the well plate in the photo field to standardize the scale. The process was repeated at every time point as previously described. The process is shown in the Fig.1

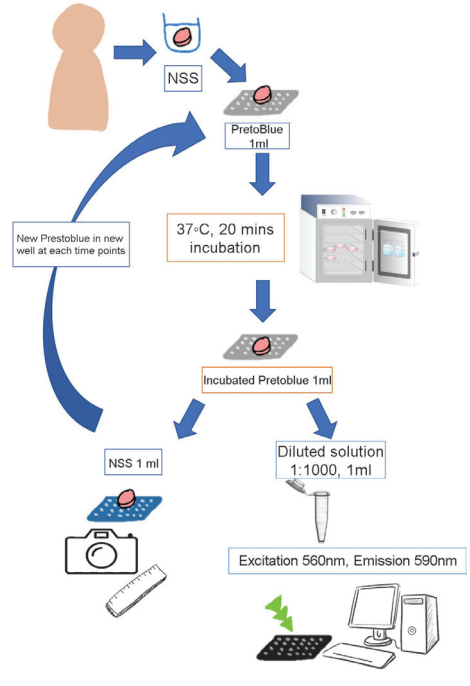

Figure 1 Experiment flow

Method flow chart. The time points of 20, 40, 60, 90, and 120 minutes were chosen to reflect the duration that clinicians and postgraduate students typically spend on periodontal surgery. Additionally, the 24-hour time point was used as a negative control, as there should be no contraction due to the absence of fibroblast activity at that time.

The area of each piece of tissue from digital images obtained from different time points was measured using ImageJ software (NIH, USA). The average graft area of each time point was calculated from 3 images taken per time point. The graft contraction was calculated for each time point and reported as the average percent original area (% of tissue area at T1) calculated from the following formula. The (*T*) was referred to as the calculated average area at each time point, and *(T1)* was referred to as the average area at the 20-minute time point (T1).

$$\%\ of\ original\ area\ = \frac{(T)}{(\mathrm{T1})} \times 100$$

The intraclass correlation coefficient of the ImageJ software measurements was calculated to be 0.86, with a 95% CI of 0.77 to 0.92, indicating good reliability.

Due to the lack of data in the literature, the sample size was estimated from an average number of patients who received periodontal soft tissue graft treatment at Periodontology clinics, Faculty of Dentistry, Mahidol University, Thailand, in a year. Since the data was not normally distributed, demographic information was reported through median and descriptions. To compare the graft contraction of the sample at each time point, Related Samples Friedman's Two-Way Analysis of Variance, with a significance level of .050 by Ranks, was used with SPSS software (IBM SPSS Statistics 26, USA).

Results

This study included 10 patients for biopsy taking and data collection. The group comprised 3 males and 7 females, with a median age of 45 years, ranging from 20 to 72 years old. One patient had asthma but was not taking any medication, while another had hyperlipidemia and had been taking atorvastatin 20mg per day for over 10 years. None of the participants were smokers. Regarding their periodontal history, 7 patients had a history of gingivitis, and 3 had a history of periodontitis, with 2 individuals in stage II and one in stage III. Ten palatal sub-epithelial CTG biopsies were collected- 4 from the anterior, 4 from the premolar, and 2 from the molar area. From our result, the different areas showed no difference in graft contraction.

From Related-Samples Friedman's Two-Way Analysis of Variance by Ranks, it was found that there was a significant difference in the graft area between 20, 40, 60, 90, 120-minute and 24-hour time points. The areas in square millimeters of harvested palatal sub-epithelial CTG at 20-, 40-, 60-, 90-, 120-minute, and 24-hour time points are presented in Table 1. The median percentages of the original area for different time points were 99.35, 96.56, 93.67, 90.89 and 97.56, as shown in Table 2 and Fig. 2. In other words, compared to the original area (T1 area) being set to one hundred percent, the samples had 0.65, 3.46, 6.33, 9.12, and 2.45 percent of contraction at 40-, 60-, 90-, 120-minute, and 24-hour time points. From the analysis of the change of graft area at each time point, it was found that there was a significant difference in the average percentages of the original area between 40-, 60-, 90-, 120-minute, and 24-hour time points (*p-value =0.03*).

Table 1 The area (mm^2) of harvested palatal sub-epithelial connective tissue graft at each time point.

	T1_20mins	T2_40mins	T3_60mins	T4_90mins	T5_120mins	T6_24Hr
Median	11.39	11.45	10.38	9.97	9.97	10.20
(P25, P75)	(8.69, 14.25)	(8.76, 13.96)	(8.26, 14.09)	(8.07, 11.61)	(7.89, 11.86)	(8.63, 11.76)

Table 2 The descriptive statistics of the average percentages of the original area for different time points

	N	Percentiles		
		25th	50th (Median)	75th
T2_T1	10	96.4775	99.3550	101.0025
T3_T1	10	91.4925	96.5550	100.5225
T4_T1	10	82.8850	93.6700	97.6675
T5_T1	10	83.0350	90.8850	99.3975
T6_T1	10	85.4925	97.5550	102.3025

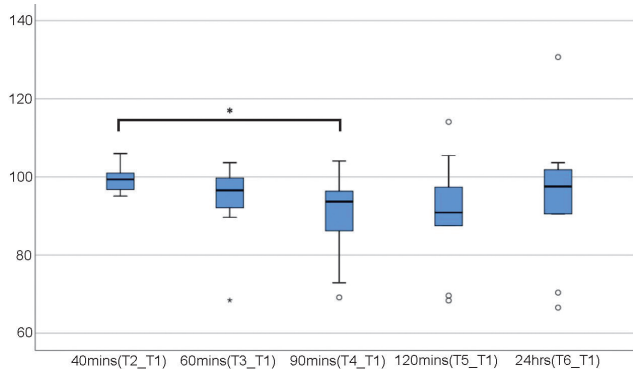

Figure 2 The boxplot with axis aligned statistics of percentage of changed area compare with the initial area at each time point and the top black line indicate the pair of significantly different time points.

Further pairwise comparisons (Table 3) showed that only one difference was found between T4 and T2, meaning that the graft area at 90 minutes decreased compared to the graft area at 40 minutes (*p-value =0.047*). No differences in the percent graft area were noted between other time points. Fig. 2 presents a boxplot of the average percentages of the original area at different time points.

Healing of soft tissue grafts typically progresses through four phases: hemostasis, inflammation, proliferation, and maturation. Hemostasis occurs first to prevent blood loss, followed by inflammation for a few days. Proliferation, which includes angiogenesis, epithelialization, granulation tissue formation, and wound contraction, takes a few weeks. Lastly, the maturation phase involves organizing connective structures like collagen and unnecessary cell apoptosis [25].

In the FGG procedure, the palatal donor wound heals at a slower rate than the CTG technique initially, but the difference becomes less significant after two to four weeks. However, patients in the FGG group report more discomfort in the first week [26].

In this study, the biopsy areas will be compared as the FGG procedure does not make any difference from the healing of the previous study, except that only one patient will be explained in the discussion part; other patients reported no significant postoperative complications, with pain scores similar between FGG (four samples) and CTG (six samples).

Patients will return weekly for infection checks and stitch removal, followed by monthly follow-ups to monitor healing, with additional visits scheduled as needed.

Table 3 Pairwise comparison of the percentage difference area at each time point compared to the original area of T1.

Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
T4_T1-T5_T1	-.200	.707	-.283	.777	1.000
T4_T1-T3_T1	1.050	.707	1.485	.138	1.000
T4_T1-T6_T1	-1.250	.707	-1.768	.077	.771
T4_T1-T2_T1	2.000	.707	2.828	.005	**.047**
T5_T1-T3_T1	.850	.707	1.202	.229	1.000
T5_T1-T6_T1	-1.050	.707	-1.485	.138	1.000
T5_T1-T2_T1	1.800	.707	2.546	.011	.109
T3_T1-T6_T1	-.200	.707	-.283	.777	1.000
T3_T1-T2_T1	.950	.707	1.344	.179	1.000
T6_T1-T2_T1	.750	.707	1.061	.289	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
a. Significance values have been adjusted by the Bonferroni correction for multiple tests.

Discussion

Based on the samples obtained; they were clinically healthy or had gingivitis or treated periodontitis. All of them completed active periodontal treatment and inflammation control. They were confirmed that they were ready for the periodontal surgery by plaque and bleeding scores.

The patient did not experience any unmanageable complications following the biopsy sample collection. Only one patient, who reported having no systemic disease, experienced prolonged bleeding that required additional suturing and Surgicel® application at the biopsy site. The patient was later referred to a physician for a blood test and was found to have high prothrombin time (PT) and high activated partial thromboplastin time (APTT). Afterward, the biopsy site recovered normally with no further complications.

Our study revealed that the graft area of the harvested palatal sub-epithelial CTG remained constant throughout that observation time, except only for the graft size at 90 minutes after harvesting. At 90 minutes, the graft area significantly decreased from the area at 40 minutes, indicating that the graft contracted significantly at 90 minutes compared to 40 minutes. To the best of our knowledge, this study is the first to report primary contraction of the CTG immediately and several minutes after detaching from the donor site. The contraction of the graft may result from fibroblast activity within the graft. The more fibroblasts function, the greater the traction forces on the graft cells, causing the graft to shrink [27].

Moreover, the stability of the graft area at different time points may be related to the presence of lamina propria in the tissue harvests. This layer serves as a robust and resilient connective tissue that is effective in resisting contraction [28]. Due to the limitations of our study, the images of the grafts taken while they were submerged in a sterile normal saline solution may exhibit some distortion. However, imaging the grafts in normal saline was necessary to assess graft vitality and to avoid tissue trauma from repeatedly transferring them in

and out of the saline. To further validate our findings, future studies with larger sample sizes are recommended to observe graft contraction over time.

The ideal area for harvesting grafts during routine procedures is from the distal canine to the second premolar region [29]. The first molar area is characterized by the thinnest mucosa, while the region surrounding the second molar has the thickest mucosa but carries a higher risk of damaging the greater palatine bundle [30]. The canine-premolar region minimizes the risk of injuring vital structures and offers sufficient palatal thickness for graft harvesting [29]. Therefore, the biopsy sample was taken from either the adjacent posterior or anterior section of the donor area to accurately reflect the application of connective tissue grafts (CTG) in clinical settings.

The normal saline solution is commonly used in clinics for immersing oral graft tissue. A microbiology study found that normal saline solution should not be stored for longer than 12 hours [31]. Additionally, the FDA has approved the normal saline solution for use with blood components [29]. In our chair-side situation, the treatment time did not exceed 12 hours; therefore, we selected this solution for our study.

The limitations

The study has certain limitations due to the relatively small sample size. As this is the first pilot study of its kind, the exact number of required samples needs further investigation. Additionally, the observation period was limited to 120 minutes, and the status of the graft area beyond this timeframe remains unknown, except at the 24-hour mark. The graft tissue samples used in this study are the same as those from another unpublished CTG vitality project; therefore, they were incubated with PrestoBlue® solution. Previous studies have confirmed that the PrestoBlue® assay is rarely toxic and is safe for cells [32, 33]. To maintain the moisture of the graft, which is vital for its vitality, the samples were immersed in a saline solution. This immersion may have contributed to some distortion in the imaging results. It is advisable to conduct a detailed histological study of the connective tissue graft (CTG) prior to transplantation to gain insights into the changes in cellular components that occur after harvesting. Such a histological study could confirm the progressive collapse of vessel structures over time and changes in cell activities that result in graft dimensional alteration. The harvested CTG in normal saline may exhibit autophagy or other mechanisms that aid its survival. Alternatively, the tissue might possess adequate energy and nutrients to sustain its functions for a limited period. Additional research is necessary to elucidate the mechanisms behind graft contraction and survival following harvesting and immersion in saline solution.

Conclusions

In this pilot study, the dimensions of palatal sub-epithelial connective tissue grafts (CTGs) remained stable for up to 120 minutes after harvesting from the palatal donor site. However, a significant contraction of the graft was observed at the 90-minute time point. After 24 hours, the graft dimensions were also consistent compared to the initial measurement. It is important to note that during actual periodontal surgery, the graft is typically stored in a normal saline solution while the surgical site is prepared or complications like bleeding are managed. Based on these findings, it is recommended that clinicians limit the storage time of CTGs in normal saline solution to less than 90 minutes to prevent graft contraction.

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Competing Interests

None declared

Ethical Approval

Ethics Committee for Human Subjects at the Institutional Review Board, Faculty of Dentistry and Faculty of Pharmacy (MU-DT/PY-IRB) on 8 Mar 2023 (No. COA MU-DT/PY-IRB 2023/024.0803).

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