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A Comparative Analysis of Collagen Fiber and Adipose Content in the Connective Tissue Procured from Different Harvesting Sites Using Different Harvesting Techniques: A Cross-Sectional Study

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Abstract

Background: Soft tissue augmentation procedures using connective tissue grafts (CTGs) produce varied outcomes due to differences in the histological composition of grafts from different sites and techniques. This study aimed to compare the histologic composition of CTG harvested by de-epithelization vs. a single-incision technique from the palate.

Methods: Forty-five subjects (aged 25-45) scheduled for mucogingival surgeries were divided into 3 groups: Group I (de-epithelialized free gingival graft), Group II (maxillary tuberosity graft), and Group III (single-incision technique). A 2 x 2 mm tissue sample was taken from the antero-superior part of the graft and analyzed using hematoxylin & eosin or Picrosirius red staining to assess collagen and adipose content under a polarizing microscope.

Results: Preoperative donor site thickness was greater in Group II than in Groups I and III ($P < .001$). Collagen area and bundle thickness were significantly higher in Group I compared to Groups II and III ($P < .05$, $P < .001$). Adipose tissue content was higher in Group III. Epithelium remnants were observed in 66.67%, 80%, and 13.3% of Groups I, II, and III, respectively ($P < .05$).

Conclusion: Fibrous tissue content was higher in superficial mucosal tissues, while deeper palatal tissues showed more adipose tissue.

Keywords: Adipose tissue, collagen, connective tissue, de-epithelialized free gingival graft, histomorphometry, maxillary tuberosity, palate

What is already known on this topic?

- *Sub epithelial Connective tissue graft provide uniform connective tissue ideal for grafting procedures.*
- *Grafts obtained from tuberosity have also shown predictable outcomes with aesthetically pleasing results.*
- *Variation in tissue composition harvested in different techniques with deepithelialized connective tissue graft harvested having significantly more connective tissue and less fatty tissue than the single incision approach.*

What this study adds on this topic?

- *The collagen bundle thickness was greater for Deepithelialized FGG.*
- *Adipose tissue content was greater in graft obtained from Single incision technique.*
- *Epithelial remnants was greater in deepithelialized FGG and tuberosity graft.*

INTRODUCTION

Periodontal plastic procedures are frequently carried out to treat anatomical, traumatic, or disease-induced defects of the gingiva, alveolar mucosa, or bone. They employ a variety of soft tissue grafting techniques, including lateral pedicle flaps, coronally repositioned flaps, free gingival grafts, connective tissue grafts (CTG), and guided tissue regeneration.[1] Covering gingival recession, increasing the width of attached gingiva, improving gingival biotype, and ridge augmentation are some uses for soft tissue augmentation.

Sub-epithelial CTG-based operations have consistently asserted the "numero uno" position in terms of mean and complete root coverage, as well as the increase of keratinized

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tissue width, despite the wide range of available techniques.[2,3] Initially introduced by Edel[4] in 1974, various modificationsof the technique have resulted in less patient pain and morbidity.[5-7]

The various modifications presented with differences in the location used, incision type, or flap designs lead to a great variability in the results. [5-7] The quantity of obtainable tissue and related patient morbidity are important factors taken into account when determining the appropriateness of a donor site.[8] The palate and the tuberosity have become the preferred regions for autograft harvesting based on these criteria.[9,10]

Amongst the most popular procedures available today, the minimally invasive technique introduced by Hurzeler et al[11] demonstrates healing by primary intention, leading to minimal postoperative discomfort during the initial stages of healing.[12] Advantages of this technique include uncompromised blood supply for the overlying flap and reduced need for suturing or postoperative stents, making it a suitable choice for procuring grafts from the palatal vault.[11,12] However, the shortcomings of the technique include reduced visibility of the site, technique sensitivity, and numerous anatomical concerns such as proximity to neurovascular bundles, precluding its use in such conditions.[11,13]

Conversely, in patients with thin gingival biotype, connective tissue thickness does not suffice for both the residual flap and the graft.[14] The residual tissue here consists primarily of epithelium, resulting in necrosis/dehiscence during healing. In such cases, the use of de-epithelialization (DE) harvested CTG would seem prudent. Such grafts can easily be procured, resulting in predictably abundant and consistently uniform connective tissue ideal for grafting procedures.[14] They also show less predisposition to postoperative shrinkage or residual tissue necrosis, with thicker recipient site gingival tissue seen postoperatively.[15]

Despite the numerous advantages of using palatal tissue, the limited thickness of mucosa and the troublesome course of the greater palatine nerves and blood vessels sometimes warrant the search for an alternative site. Thus, sites such as the maxillary tuberosity could be used to procure thicker grafts, additionally reducing patient discomfort from a second surgery.[16] Adequate thickness of keratinized tissue along with the absence of major blood vessels marks it as an appropriate alternative.[17] Moreover, clinical outcomes obtained from using tuberosity tissue have been shown to be predictable with an aesthetically pleasing outcome.[18]

A recent cross-sectional study was performed on cadavers to assess fibrous and fatty tissue content in the palatal grafts taken from different locations and depths of tissue. The authors reported high inter-individual differences; regardless of the harvesting site, there is variation in the hard palate's relative tissue composition, with DE-harvested CTG having significantly more CT and less fatty tissue than the single incision approach.[15]

A thorough literature search revealed a lack of similar comparative studies in humans. Thus, the present study aims to assess and compare the histologic composition of CTGs harvested by DE and single-incision in the palate, and those harvested from the tuberosity, taken from 45 subjects scheduled to undergo mucogingival surgeries.

MATERIALS AND METHODS

Study Population

A cross-sectional study design was proposed for the research. Ethical committee approval was received from the Institutional Ethical Committee and Review Board of Bapuji Dental College and Hospital, Davangere, Karnataka, India (Approval no: BDC/Exam/87/2016-17), Date: 29.06.2016). and written consent was obtained from each patient. A total of 45 subjects (between the age group of 25-45 years) were selected for the study.

Patients scheduled to undergo mucogingival surgeries and subjects with proper oral hygiene and overall health were included. Participants with preexisting medical conditions such as diabetes mellitus, scurvy, scleroderma, rheumatoid arthritis, systemic lupus erythematosus, and other collagenopathies, as well as those wearing palatal prostheses, were not allowed to participate in the study.

The participants were divided into 3 groups: Group I (DE free gingival graft), Group II (maxillary tuberosity graft), and Group III (single-incision technique), with 15 patients in each group. The groups were organized based on the site and/or technique used for harvesting connective tissue. Clinical measurements included soft tissue parameters like the thickness of the donor site, measured using an endodontic needle with a silicon disc inserted perpendicular to the palate, as previously described. All the grafts were harvested as per the previously described standard techniques (Figures 1-3).[3-8]

The study was conducted independently by the operator (IN). Due to the nature of the study, the operator could not be blinded, but allocation was not known until the start of the procedure. The researchers who performed collection of material and analysis (T.A.B., R.S.) were blinded. A minimum of 2 x 2 mm of tissue was taken from the antero-superior portion of the procured graft.

Histochemistry and Image Analysis

A uniform approach was used to prepare the tissue samples from each of the three groups. For 24-48 hours, the samples were fixed in a 10% neutral buffered formalin solution. These samples underwent additional processing with increasing grades of alcohol, chloroform, and paraffin wax embedding. After being deparaffinized in a hot air oven, they underwent 2 changes of xylene. Following a thorough rinse, haematoxylin & eosin staining was applied.

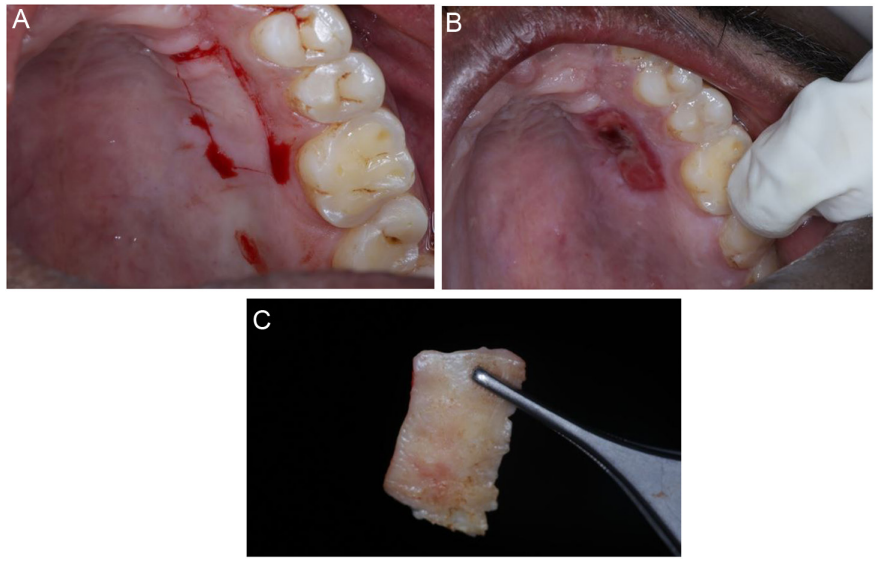

Figure 1. (A) Harvesting FGG from the palate. (B) Donor site. (C) De-epithelialized FGG obtained for study.

After routine staining, another specimen was subjected to the above procedures, hydrated, and then incubated with Picro-sirius Red Stain solution for 1 hour at room temperature. Finally, it was rinsed, dehydrated, and mounted with DPX mountant (Fisher scientific) (Figure 4). Picro-sirius Red Stain Solution was prepared by the addition of 100 mL saturated aqueous picric acid to 0.1 gram of Sirius red F3BA, as described previously (Direct Red 80, C.I. 35780; Sigma-Aldrich, St. Louis, MO, USA). [18]

Collagen and Adipose Tissue Quantification

Picro-sirius red-stained sections were observed under a polarizing microscope. All the observations were made by 2calibrated, double-blinded examiners who were unaware of the nature of the experimental groups and each other's assessment. Three fields from each slide were selected, and thickness of collagen fibers was measured in each field (in microns) using image analyzer software (ProgRes CapturePro 2.8.8 software, Jenoptik, Jena, Germany). Additionally, their polarizing color was noted.

The area occupied by collagen fibers was calculated (in square microns). For all samples, 3 fields per tissue section were analyzed. Similarly, the areas covered by fatty/glandular tissue were calculated (in square microns). Subsequently, the means of all these parameters were calculated. The area occupied by collagen fibers or adipose tissue was presented in the form of percentages. Also, in the presence of remnant epithelium, the depth of the lamina propria was measured. [19]

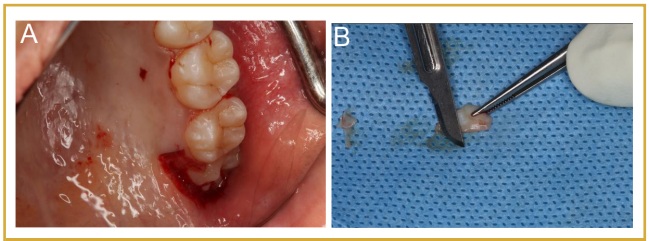

Figure 2. (A) FGG harvested from maxillary tuberosity. (B) FGG de-epithelialized.

Statistical Analysis

The obtained values were subjected to statistical analysis. The statistical analysis was performed using IBM SPSS Statistics version 20 software (IBM SPSS Corp.; Armonk, NY, USA). The descriptive statistics were presented as mean and mean SD. One-way ANOVA followed by Post Hoc Tukey's test was used for the comparative evaluation of the area covered by collagen and donor site tissue thickness. The Kruskal-Wallis test compared the thickness of collagen bundles between different groups. Intergroup comparison of the area covered by adipose tissue was done by Mann–Whitney U-test and Fisher's exact test compared the presence of epithelial remnants among the different groups.

$P \leq .05$ was considered statistically significant, $P < .001$ was considered statistically highly significant, and $P > .05$ was considered statistically non-significant.

RESULTS

The preoperative thickness of the donor site was higher in Group II compared to Group III and Group I. The difference in the values between Groups I and II and Groups II and III was found to be statistically highly significant ($P < .001$). However, the mean difference in tissue thickness was non-significant for Groups I and III ($P = .18$) as seen in Table 1.

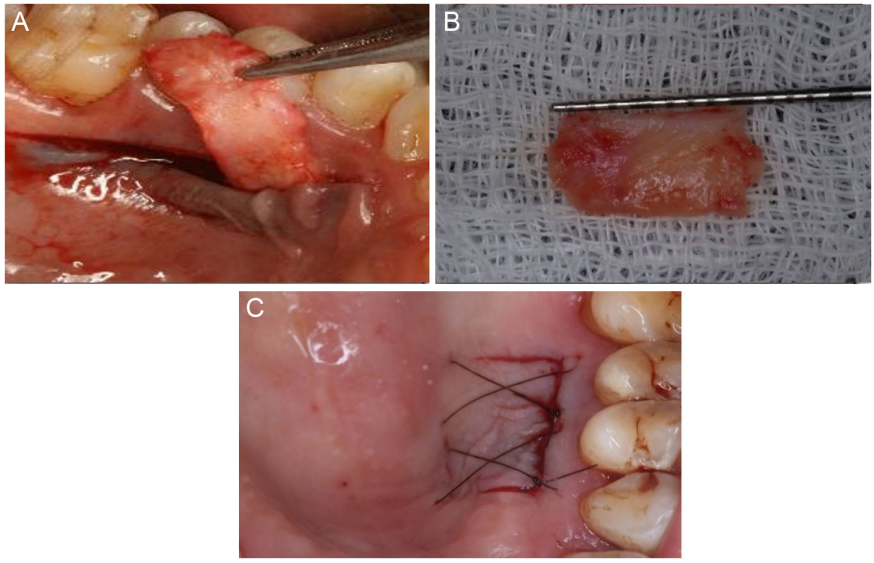

Figure 3. (A) Sub-epithelial connective tissue graft harvested by single incision technique. (B) Connective tissue graft harvested. (C) Donor site sutured.

The area occupied by collagen was greater for Group I as compared to Groups II and III. The mean difference between Groups I and III and Groups II and III was found to be statistically significant ($P < .05$). However, the mean difference in area occupied by collagen was statistically non-significant for Groups I and II ($P = .98$) as seen in Figure 5.

The collagen bundle thickness was greater for Group I as compared to Groups II and III. The mean difference in the collagen bundle thickness was found to be statistically highly significant ($P < .001$) between Groups I and Group II, Group I and Group III, and Group II and Group III, as observed in Table 1.

The adipose tissue content was greater for Group III as compared to Groups I and II. The mean area difference in the adipose tissue content between Group I and Group II, Group I and Group III, and Group II and Group III was statistically significant ($P < .05$), as observed in Figure 5.

Remnants of epithelium were observed in 66.67%, 80%, and 13.3% of Group I, Group II, and Group III sections, respectively. Conversely, 33.3%, 20%, and 86.67% of Group I, Group II, and Group III sections, respectively, had no remnants of epithelium. Statistically significant differences were observed between all the groups ($P < .05$).

DISCUSSION

Over the years, various soft tissue augmentation techniques have been associated with different success rates. Such variations seen in the treatment outcomes have been attributed to dissimilarities in the histological characteristics of the tissue procured from different harvesting sites using different techniques.[8,13,20] After an extensive literature search and a paucity of information regarding the same, we conducted a cross-sectional study on patients to assess, evaluate, and compare the fibrous and adipose content of grafts.

In the present study, the thickness of tissue obtained from the tuberosity was found to be higher compared to the palatal tissue. Also, the thickness of mucosa for both single incision and DE FGG was found to be similar. This finding is consistent with an earlier study by Cortellini and coworkers, where the palatal masticatory mucosa thickness at the area of second molars and premolars was observed to be the second thickest, after the tuberosity.[21,22] As a result, tuberosity tissue can be split longitudinally "like a book" in order to obtain longer grafts.[17] Thus, clinically it could be used when longer grafts are required, such as multiple recession coverages or long span soft tissue augmentation.

Single incision technique would preferentially be done in cases with thicker biotype, resulting in sufficient residual tissue thickness even after subepithelial connective tissue graft (SCTG) harvestation.[23] Thin biotype typically warrants the use of DE-FGG. In this, an FGG is retrieved and DE extra-orally. This leaves the periosteum intact, thus ensuring no disruption of blood supply to the donor site. It also minimizes the chances of tissue necrosis and postoperative discomfort. This study has demonstrated lesser preoperative tissue bulk in the single incision group; however, the difference was statistically non-significant. Additionally, postoperative discomfort has recently been evaluated by Zucchelli et al[8] and they came to the conclusion that postoperative analgesic intake was controlled by the height and depth of the withdrawal rather than the type (primary vs. secondary) of palatal wound healing.

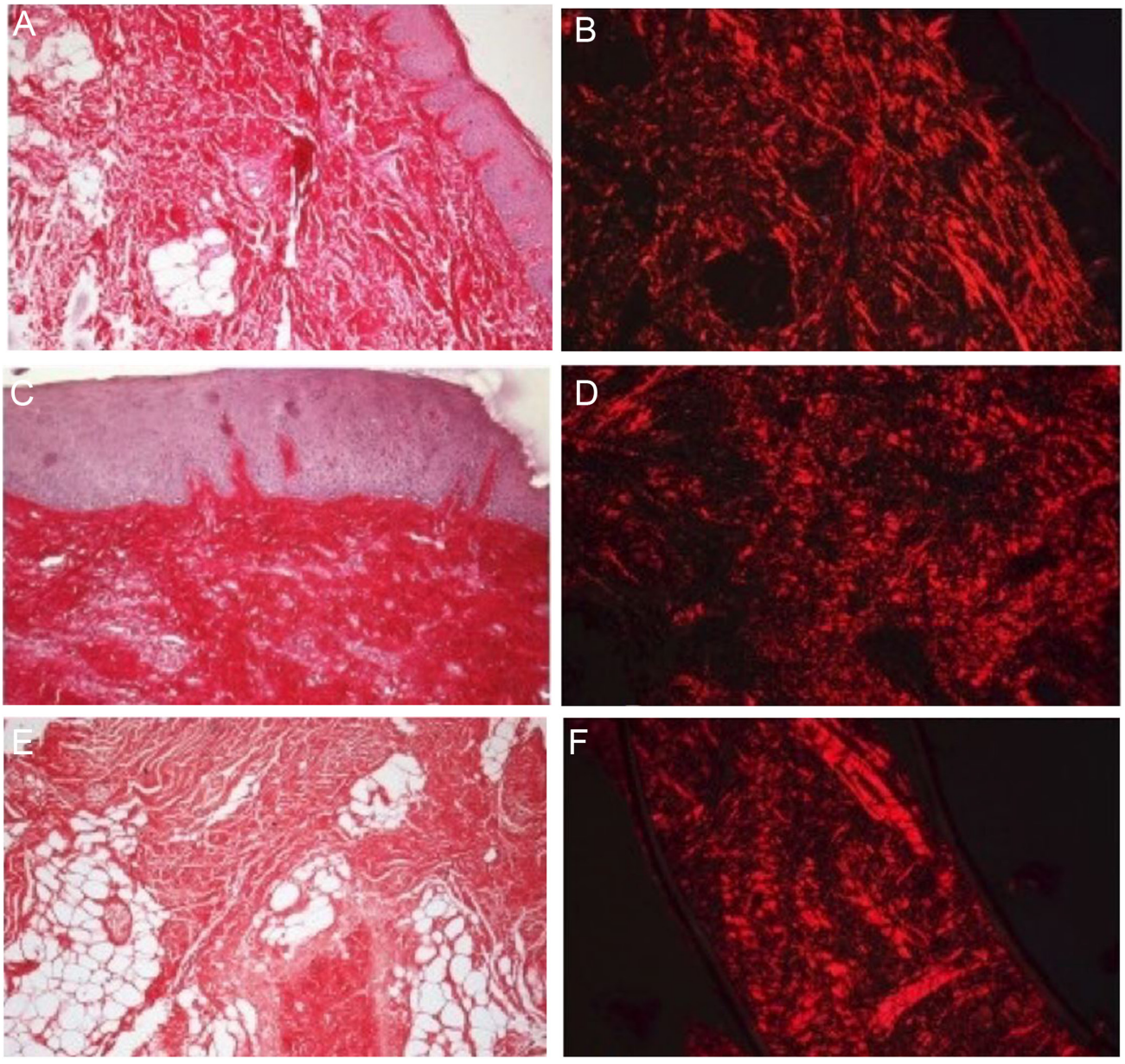

Figure 4. (A) Figure depicting Picro-sirius staining at 10x in Group I samples showing collagen fibers under a research microscope. (B) Figure depicting Picro-sirius staining at 10x in Group I samples showing collagen fibers under a polarized microscope. (C) Figure depicting Picro-sirius staining at 10x in group II samples showing collagen fibers under a research microscope. (D) Figure depicting Picro-sirius staining at 10x in Group II samples showing collagen fibers under a polarized microscope. (E) Figure depicting Picro-sirius staining at 10x in Group III samples showing collagen fibers under a research microscope. (F) Figure depicting Picro-sirius staining at 10x in Group III samples showing collagen fibers under a polarized microscope.

Greater depth of blade insertion increased the chances of severing a large-sized nerve/vessel, causing greater pain.[8] This finding is in stark contrast to the presumed advantages of procuring a CTG over FGG. It increases the clinical acceptance and applicability of performing free gingival grafts.

In the present study, significant inter-group variations in the area covered by collagen were observed, with DE-FGG and tuberosity grafts containing greater collagen content. These study results are in agreement with those by Bertl and co-workers, who have also demonstrated lower fibrous tissue content in split-flap CTGs than in tissue procured using

Table 1. Intergroup Comparison of the Preoperative Tissue Thickness, Thickness of Collagen Bundles, and Presence of Epithelial Remnants

Groups	Preoperative Tissue Thickness			Thickness of Collagen Bundle (Kruskal-Wallis Test)			Epithelial Remnants			
n = 15 each	Mean ± SD	Comparison of Groups (Tukey Post hoc test)	*P*	Mean ± SD	χ2 Value (*df*)	*P*	Present	Absent	χ2 Value (*df*)	*P*
I	2.67 ± 0.52	I vs. II	<.001*	9.60 ± 2.78	13.02(2)	.001*	5 (33.3%)	10 (66.7%)	7.22(2)	.027*
II	3.73 ± 0.62	I vs. III	.18(NS)	8.37 ± 2.30			3 (20.0%)	12 (80.0%)		
III	3.00 ± 0.33	II vs. III	.001*	5.85 ± 3.05			13 (86.7%)	2 (13.3%)		

*$P < .05$. Statistically significant.
$P > .05$. Non-significant, NS.

DE- FGG.[13] Dellavia et al[20] compared the collagen content of the palate (using CTGs) and the tuberosity area and observed non-significant differences between the two. In contrast, we observed that tuberosity and DE-FGG have a higher amount of collagen fibers compared to SCTG.

The quantity of fibrous connective tissue has been correlated clinically to a decreased amount of postoperative shrinkage, increased buccal gingival tissue thickness, and ease of clinical handling.[6] Thus, a higher quantity of collagen in a soft tissue graft would appear to result in a more desirable outcome. Keeping these results in mind, it would be logical to assume DE-FGGs or tuberosity tissue would better manage ridge augmentation techniques or coverage of recessions. Moreover, a deeper recession would most likely benefit from the use of a partially epithelialized FGG. Here, there would be fewer chances of necrosis of the tissue lined by epithelium when exposed to the oral environment.[4]

We also observed significant differences in collagen bundle thickness ($P > .05$) between the different groups. Among the groups, CTGs obtained by single incision technique showed minimum bundle thickness, which is consistent with the observation that as submucosal depth increases, adipose content also increases. Additionally, the fibrous tissue is more loosely arranged in the palatal aspect of the submucosa.

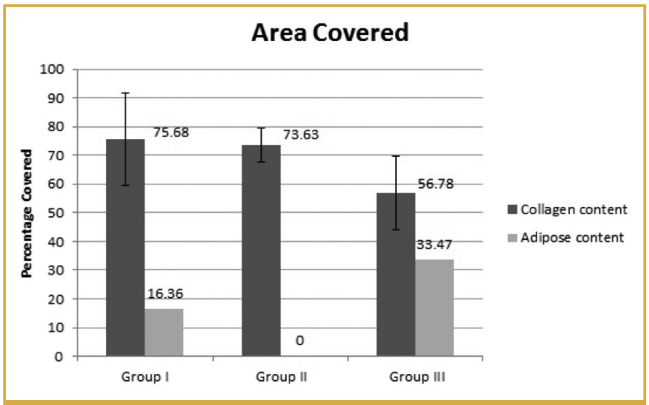

Figure 5. Descriptive statistics for area covered by collagen and adipose content.

Contrariwise, DE-FGG and tuberosity are derived from the more superficial sections of mucosa with a clear lamina propria and well-organized collagen bundles. This endows them with properties such as clinical ease of handling and decreased post-operative shrinkage. Clinically, this would be an excellent indication for ridge augmentation or root coverage procedures.

When compared among the three groups, the adipose tissue content of the tuberosity was found to be much less than that of the tissues procured from the palate, i.e., either single incision technique CTG or DE-FGG. This may clinically relate to greater incidences of scarring and hyperplastic reactions at tuberosity grafted sites when tissue from the tuberosity is procured.[24] Moreover, a significant difference was noted in the adipose content of DE- FGG vs. CTG obtained by single incision technique.[14] It is also important to observe that the majority of patients have a dense layer of glandular and fatty tissue beneath a thin layer of connective tissue in the palatal premolar region.

Thus, in a thinner biotype, it may be beneficial to procure a DE-free gingival graft (FGG) over a CTG graft as adipose tissue incorporated in a graft may act "as a barrier both to diffusion and vascularization."[7] Additionally, adipose tissue shrinks more and is more readily compressed by the flap that covers it.[18] In this respect, the CTG would appear the least desirable option when the main aim is to provide bulk to tissue, such as in cases of ridge augmentation.

Previous authors, including Arcidiacono et al, have found tuberosity tissue to be denser in comparison to palatal tissue.[10] This may clinically lead to dimensional stability and hyperplastic reactions of the graft post-operatively.[24] In contrast to an SF-harvested CTG, which comes from a deeper aspect of the palate, Bertl et al[13] have demonstrated that a DE-harvested CTG, which comes from a more superficial aspect of the palate, has significantly less fatty/glandular tissue. This study also reported a significant difference in the amount of adipose content between the 2.

Higher numbers of samples of DE-FGGs and tuberosity tissues contained remnants of epithelium (66.67% and 80% respectively), whereas only 13.3% of CTG samples had epithelial remnants. Previously, Zuhr et al[12] demonstrated a persistence

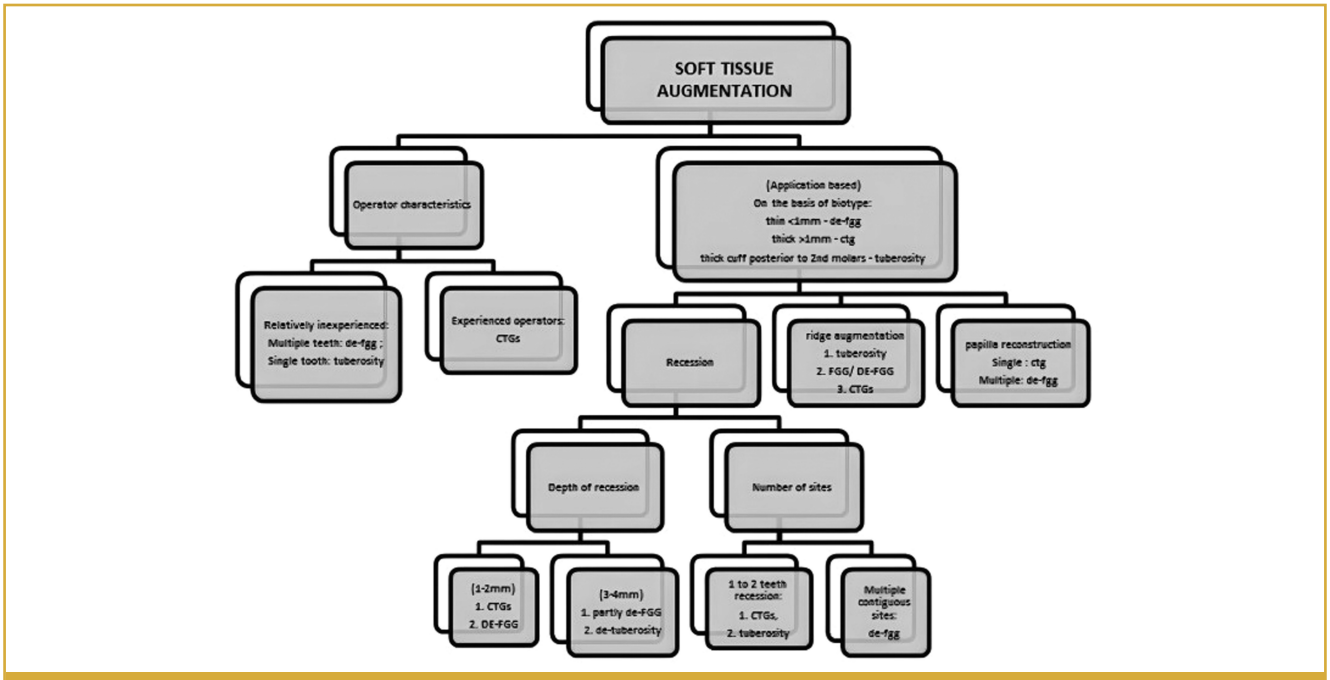

Figure 6. Decision tree.

of epithelial remnants in DEFGGs that could make scar tissue more likely to develop. This is due to the papillary inter-locking between the epithelium and the lamina propria.[10] Authors have also proposed that there may be a risk of epithelial cyst formation in such cases.[25]Clinically, this would correlate to a reduced applicability of DE tissues in aesthetic sites.

Since the absolute indications for the choice of site/technique for soft tissue augmentation have not been demarcated previously and based on the various histological and clinical findings of this study with support from literature, we propose a decision tree for selection with respect to various applications of soft tissue (Figure 6). One of the first parameters here includes the operator skills, wherein the single incision technique is generally found to be more technically demanding and requires some amount of clinical experience. Alternate use of DE-FGG[26] or tuberosity tissue is indicated in such cases. The procedure is easy and practical and does not require a significant learning curve or additional surgical instruments.[26] Moreover, macro and microsurgical approaches are equally efficient in CTG harvesting, but in this study, the macrosurgical approach is used.[27]

Next, we have divided the indications on the basis of applications, i.e., recession coverage, soft tissue augmentation, or papillary reconstruction. All these procedures additionally need a preoperative biotype assessment for a better clinical approach. Thus, patients with a thinner biotype would benefit from a DE-FGG approach, whereas a thick cuff posterior to the maxillary molars would indicate a tuberosity approach. Root coverage can be subdivided on the basis of the depth of recession and length of the graft required. Ridge augmentation would benefit mostly from thicker tissue, which is found in the tuberosity region.

Our cross-sectional study has compared the connective tissue composition of grafts procured from different sites using different techniques. However, these findings need to be correlated clinically with the post-operative recipient site changes in order to better assess their advantages in the various clinical situations.

CONCLUSION

Hence, within the constraints of the current investigation, it may be said that tissues obtained from DE-FGG and tuberosity have higher and thicker collagen content, whereas tissues from tuberosity present with lower adipose content. Also, remnants of epithelium occur more frequently in DE-FGG and tuberosity grafts. Hence, DE-FGGs and tuberosity could be used in cases of ridge augmentation where bulk of tissue would be required, whereas soft tissue coverage procedures would benefit better from a single incision technique.

Data Availability Statement: The data that support the findings of this study are available upon request from the corresponding author.

Ethics Committee Approval: Ethical committee approval was received from the Institutional Ethical Committee and Review Board of Bapuji Dental College and Hospital, Davangere, Karnataka, India (Approval no: BDC/Exam/87/2016-17), Date: June 29, 2016).

Informed Consent: Written informed consent was obtained from patients who agreed to take part in this study.

Peer-review: Externally peer reviewed.

Author Contributions: Concept – I.N., R.S., R.T.; Design – I.N., T.K.; Supervision – T.K., R.T.; Resources – I.N., T.K.; Materials – I.N., T.K.; Data Collection and/or Processing – R.S., I.N.; Analysis and/or Interpretation – R.S., J.G.; Literature Search – T.M., R.T.; Writing Manuscript – I.N., J.G.; Critical Review – R.S., T.M.

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Declaration of Intere: The authors have no conflict of interest to declare.

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