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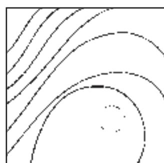
Viability Analysis of Subepithelial Connective Tissue Grafts Subjected to a Mechanical Expansion Process: A Histologic Study in Dogs



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The aim of this study was to histologically evaluate the viability of mechanically expanded subepithelial connective tissue grafts. Sixteen samples of palatal connective tissue were collected from eight beagle dogs. Half of the samples were subjected to the expansion procedure and used as subepithelial grafts in the canine region, and the samples not subjected to expansion were grafted at the contralateral side. After 60 days, biopsies were collected and examined histologically by light and confocal laser microscopy and immunohistochemically with anti-CD31 antibody for endothelial cells. There were no significant differences between the control and test groups. It was concluded that this new method to expand the area of connective tissue grafts was not only viable biologically, but also decreased surgical risks without increasing processing time.
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Since the 1960s, grafting techniques have been used to improve health, function, and esthetics in restorative dentistry.[1] Several studies have demonstrated that the presence of keratinized mucosa, as well as its quality (thickness) and quantity (width), around teeth and implants are determining factors for successful treatments and oral health.[2,3]

The use of connective tissue grafts to correct periodontal and peri-implant defects, whether functional or esthetic, has increased and is now a routine procedure in dental practice.[2–6] There are, however, unfavorable or limiting factors to this technique, such as hemorrhage from the donor site and insufficient donor tissue. Hemorrhage and insufficient donor tissue are related to the area and volume of the donor site. Degeneration of the epithelium that covers the donor site may lead to bone exposure.[7] Anatomical characteristics of the palatal region, such as shape and size, determine the amount of graft that can be harvested. Another limiting factor is the location of the

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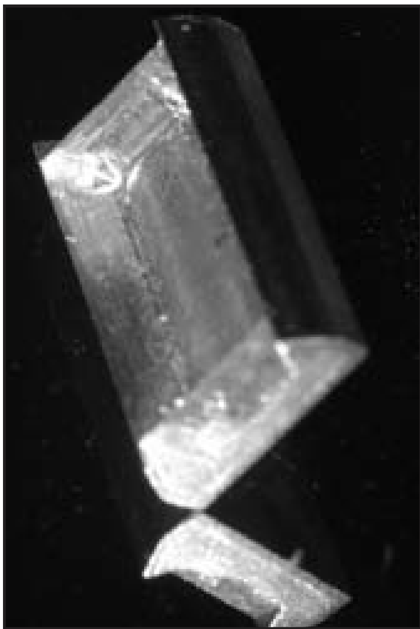

Fig 1 *Incision template (2 × 1 cm).*

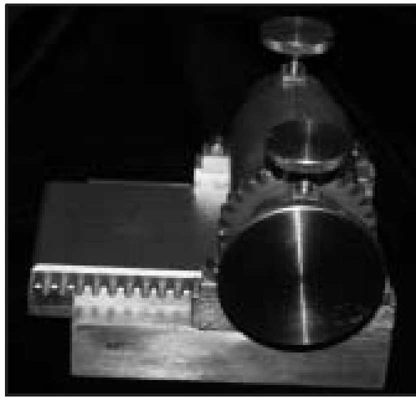

Fig 2 *Tissue expansion device.*

Fig 3 *Expansion of a connective tissue sample.*

greater palatine artery.[8,9] Results from studies using cadavers have determined that the premolar region is the thickest in the palate for the harvesting of grafts, and if the size of grafts that can be removed from this region is insufficient, tissue should be harvested from the contralateral side.[9] Although the palatal region is the most commonly used donor site, tissue harvested from other sites, such as the retromolar region and edentulous areas, as well as tissues resulting from gingivectomy can be used, but the amount of tissue available from these sources is limited.[10]

The objective of this study was to histologically evaluate sites grafted with palatal connective tissue subjected to a mechanical expansion procedure aiming to minimize the amount of harvested tissue by increasing the area of tissue grafts.

Method and materials

Eight beagle dogs approximately 10 months old and weighing 10 to 18 kg were selected for this study. Prior to the surgical procedure, each dog received an intramuscular injection of atropine sulfate (0.44 mg/kg; Atropinon 0.50 mg, Hipolabor Farmacêutica). After 10 minutes, xylazine (3 mg/kg; Rompun, Bayer) and ketamine chlorohydrate (16 mg/kg; Francotar 1.0 mg, Virbac) were administered intramuscularly.

One sample of connective tissue was surgically removed from each dog. To standardize the sample size, a rectangular incision template measuring 2 × 1 cm was used (Fig 1). The template was pressed against the palatal mucosa resulting in partial incisions; the incisions were completed using a scalpel (blade 15C, SurgiBlade). Samples were harvested from the left and right sides of the palate from a region close to the median raphe and first molars of the dogs.

After harvesting, samples were cut into halves and distributed randomly into control and test groups. The samples from the test group were compressed gradually at 0.5-mm intervals with a tissue expansion device (Fig 2) developed by the Departments of Engineering and Stomatology, Federal University of Santa Catarina, Florianópolis, SC, Brazil, until they reached a final thickness of approximately 0.75 mm (Fig 3). Samples from the control group were not subjected to the expansion procedure. Tissue grafts from both groups were then placed (without coronal displacement, using only sulcular incisions and graft splitting, and keeping the periosteum attached to the bone)

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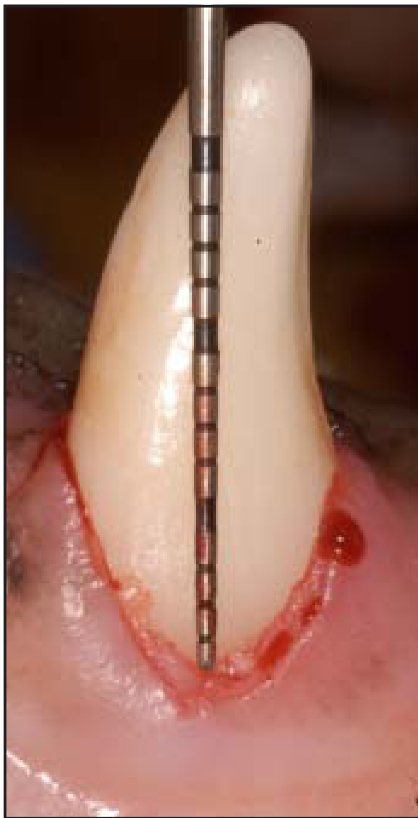

Fig 4a *Gingival recession created in the canine region.*

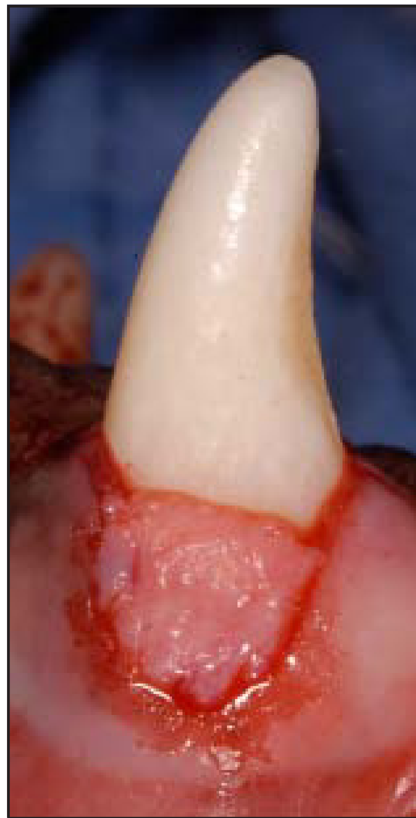

Fig 4b *Graft tissue positioned on the recession defect.*

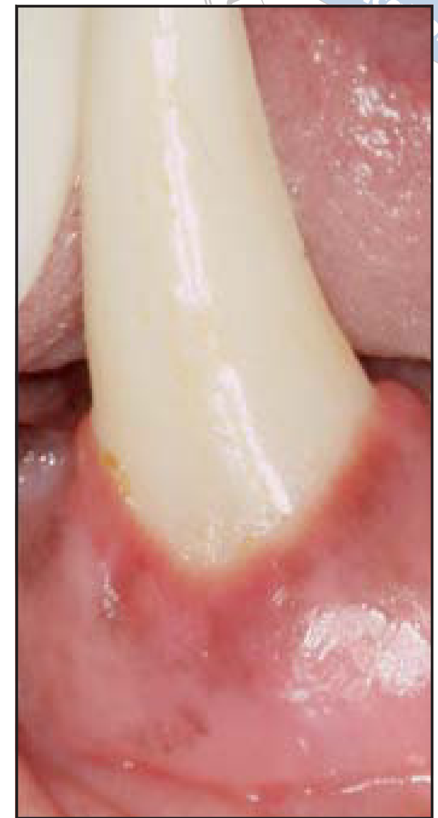

Fig 4c *Postoperative view 60 days after graft placement.*

into the mandibular canine region of the same animal from which the tissue graft was harvested. Recessions measuring 5 × 5 mm were created in this region prior to preparation of the recipient beds (Fig 4).

Following surgery, each dog received an analgesic and anti-inflammatory agent, flunixin meglumine (1.1 mg/kg intramusculary; Banamine 10 mg injectable, Schering Plough), for 7 consecutive days. The animals also received an antibiotic (Pentabiótico Veterinário Pequeno Porte 1,200,000 U, Fort Dodge Saúde Animal) for 10 days (24,000 U/kg per day intramusculary).

Histologic analysis

After 60 days, a biopsy of the grafted area was performed using a biopsy punch (5 mm in diameter). The tissue samples were fixed in 10% formaldehyde for 72 hours, dehydrated in an ascending series of alcohol concentrations (70% to 100%), and embedded in Paraplast (Leica). The embedded samples were cut into 5-µm semiserial sections and stained with hematoxylin-eosin (h&e).

Histologic analysis using h&e staining consisted of the qualitative comparison of tissue samples from the test and control groups with respect to tissue changes, such as the presence of inflammatory infiltrate (Fig 5).

Histomorphometric analysis

A confocal scanning laser microscope (TCS SPE, Leica) and a light microscope (Axioskop II, Carl Zeiss) coupled to image-processing software (KS 300 Imaging System release 3.0, Carl Vision) were used for the analysis of histomorphometric parameters. Because of the fluorescence properties of eosin, morphometric analysis of sections stained

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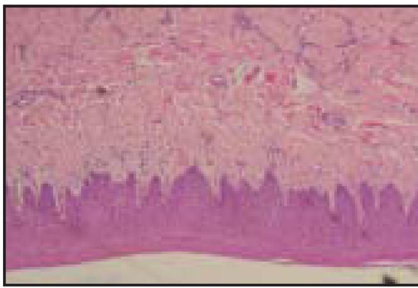

Fig 5 (left) *Light microscopy of h&e stained section from the test group. Note the absence of inflammatory infiltrate (original magnification ×10).*

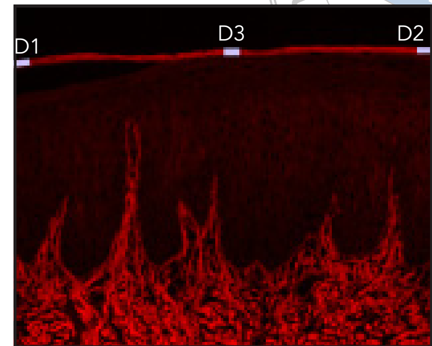

Fig 6 (right) *A confocal microphotograph showing the positions where the thickness of the keratin layer was measured (D1, D2, and D3).*

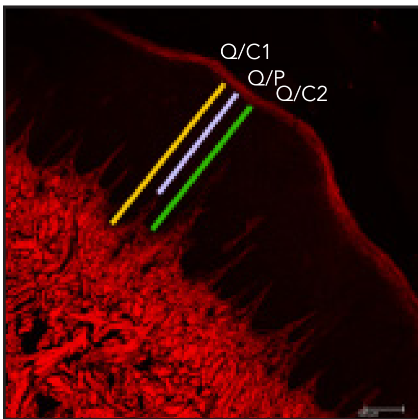

Fig 7 (left) *Confocal microphotograph showing the positions where the thickness of the epithelium was measured (Q/P, Q/C1, and Q/C2).*

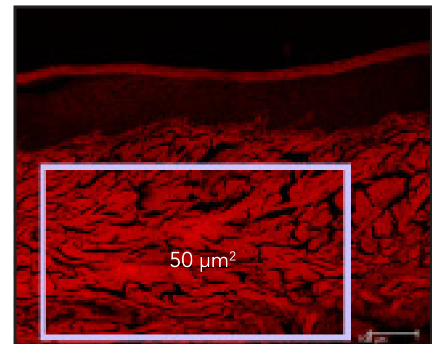

Fig 8 (right) *Area selected from a histologic section measuring 215.371 μm² for collagen fiber measurements.*

with h&e was conducted using the confocal laser scanning microscope at 10× magnification.

Comparative analysis of the thickness of the epithelium and measurements of the collagen fiber area in the connective tissue subjacent to the epithelium were performed as indicated.

Keratin thickness

To quantify the keratin thickness in the samples, three points (D1, D2, and D3) were selected on the keratin layer. The keratin thickness was calculated from the arithmetic mean of the thickness at these three points (Fig 6), where D1 is the keratin thickness at the left corner of the section, D2 is the keratin thickness at the right corner of the section, and D3 is the keratin thickness at the center of the section.

Thickness of the epithelium

To quantify the thickness of the epithelium, the largest distance between the connective papilla and keratin was identified on the histologic section; then, three thickness measurements were taken at this position. The epithelium thickness was defined as the arithmetic mean of these three values (Fig 7), where Q/P is the thickness measured from the beginning of the keratin layer to the center of the connective papilla, Q/C1 is the thickness measured from the beginning of the keratin layer to the center of the left epithelial ridge, and Q/C2 is the thickness measured from the beginning of the keratin layer to the center of the right epithelial ridge.

Area of collagen fibers in the subepithelial connective tissue

To measure the area covered by collagen fibers in the connective tissue subjacent to the epithelium, an area of 50 μm² was selected from the center of the histologic section. The total area of this section measured 215.371 μm² (Fig 8).

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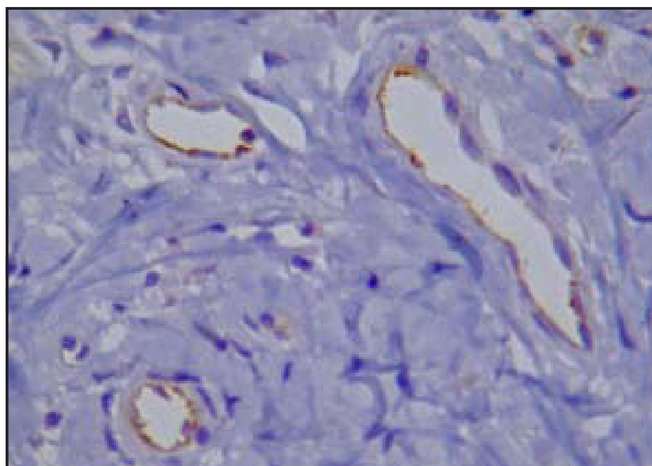

Fig 9 *Light microphotograph of a section from the test group. Note the reddish color of blood vessels stained with anti-CD31 antibody (original magnification ×40).*

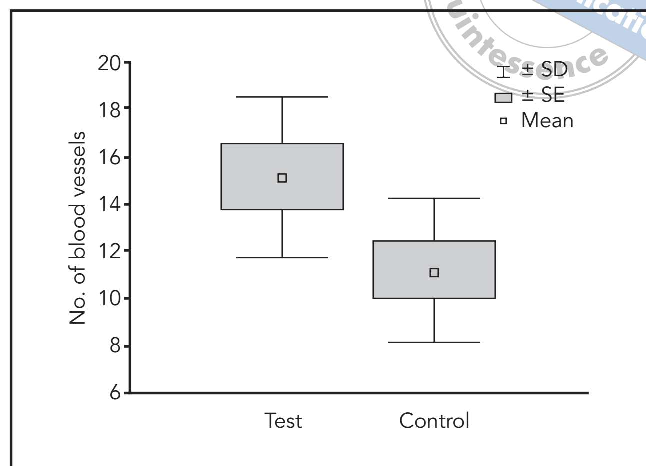

Fig 10 *Comparison of the number of blood vessels in the control and test groups. There were no significant differences between groups (P > .5).*

After selecting the area, the image was analyzed using the KS 300 Imaging System 3.0 software, and the amount of collagen fibers present in the connective tissue subjacent to the epithelium was measured.

Immunohistochemical analysis

For the immunohistochemical analysis, 3-μm-thick sections were prepared from tissue samples fixed in 10% formaldehyde and embedded in paraffin. The sections were mounted on glass slides, previously cleaned and degreased, coated with 3-aminopropyltriethoxysilane (Sigma Chemical). The sections were stained using the streptavidin-biotin complex technique with monoclonal anti-CD31 antibody (JC70A, Dako) for the identification of endothelial cells, after antigen recovery with 0.4% trypsin and antibody incubation at 4°C for 18 hours. Cell counting was then performed using light microscopy at 40× magnification (Fig 9). The brownish structures were stained with the monoclonal anti-CD31 antibody for endothelial cells.

Statistical analysis

The results were evaluated using analysis of variance and the Student *t* test. The statistical software used was Estatistica release 6.0 (StatSoft).

Results

There were no significant differences regarding the qualitative analysis of tissue changes between control and test groups, as observed by h&e staining. Also, there were no significant differences in the results of the histomorphometric analysis of the thickness of the keratin layer and epithelium or the amount of collagen between control and test groups.

Figure 10 shows that there were no significant differences between control and test groups regarding the number of blood vessels stained with monoclonal anti-CD31 antibody for endothelial cells ($P > .05$).

The actual increases in the area of the connective tissue grafts subjected to the expansion procedure (test group) are listed in Table 1. The mean value of the area was obtained from measurements made before and after expansion; the connective tissue area increased approximately 158% (Fig 11).

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Table 1 Dimensions (mm) of the connective tissue grafts before and after the expansion procedure

	Dog							
	1	2	3	4	5	6	7	8
Before	5.5 × 6.0	6.0 × 5.0	8.0 × 6.0	4.5 × 9.5	8.5 × 7.0	7.5 × 8.0	5.5 × 6.0	6.0 × 8.0
After	10.5 × 8.0	11.0 × 7.0	11.0 × 9.0	13.0 × 11.0	13.5 × 11.5	11.0 × 10.0	10.0 × 9.0	9.5 × 15.0
% expansion	154.5%	156.6%	106.3%	234.5%	160.9%	83.3%	172.7%	196.9%

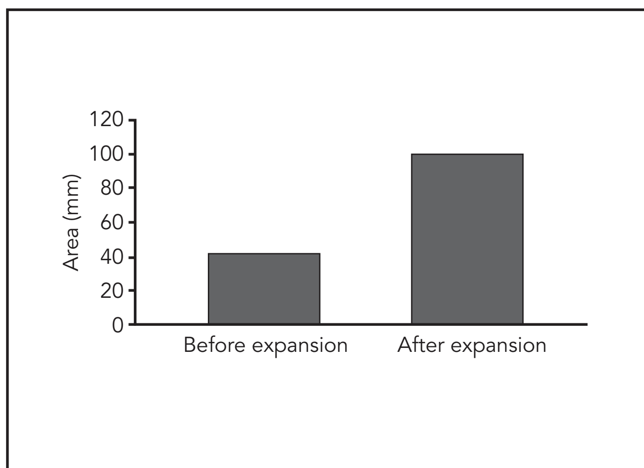

Fig 11 *Mean coverage area of connective tissue grafts before and after expansion.*

Discussion

Both the quality and quantity of keratinized mucosa are determining factors for maintaining the health and esthetics around teeth.[11] The absence of tissue with these characteristics results in an inadequate marginal seal of the mucosa because of the increase in mobility, which may promote the subgingival colonization by bacteria.[12] Other factors, such as impermeability and the mechanical protection provided by the masticatory mucosa, which protects the periodontium from chemical and physical stress, must also be considered.[13] Because of these factors, adequate quality and quantity of keratinized mucosa inhibits the inflammatory process, preventing loss of attachment and gingival recession.[14]

Root coverage with subepithelial connective tissue grafts has been used for several decades.[15] The major limitations of this technique are related to the risk of injury to the vascular/nerve plexus in the donor bed.

Blood vessel generation and soft tissue augmentation can occur both directly and indirectly. Indirect forms of soft tissue gain occur through osteogenesis distraction and orthodontic traction. These treatments promote an increase in bone volume and, indirectly, a gain in the soft tissue. These techniques have the advantage of maintaining the integrity of blood vessels, increasing local irrigation, and also allowing soft tissue gain, especially in height, better than direct techniques. Among the direct techniques, it may be beneficial to highlight the conjunctive subepithelial graft, which has the advantage of low risk of bleeding and has been highly publicized in peri-

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odontics, although it has limitations in the quantity of donor tissue available, postoperative discomfort, and the need to section an area of blood vessels larger than the area to be grafted, thereby determining that the nutrition of the original tissue is grafted by plasma diffusion. The indirect techniques should only be undertaken if a gain in bone structure and soft tissue is needed because cases involving orthodontic traction can result in crown and tooth wear and loss of periodontal support. Osteogenesis distraction has precise indications and risks of failure, with a high rate of bone loss remaining.

The use of large amounts of connective tissue renders the subepithelial connective tissue graft technique impracticable or leads to a high postoperative morbidity.[16] Therefore, the smaller the amount of harvested tissue, the smaller the risks involved. The prospect of being able to harvest a small amount of tissue and expand it before grafting has motivated research on this topic.[17] A study by Pini Prato et al[17] exemplified this type of research in their study consisting of the in vitro expansion of a sample of gingival tissue, which was then used as a graft. However, this technique was time consuming and expensive, and it was not tested for cytotoxicity and genotoxicity.[17] Another technique, which consists of striking the tissue with the handle of an Ochsenbein chisel, has been used by clinicians. However, because of the lack of studies evaluating this technique, its clinical and scientific efficacy is questionable.

The current study serves as a scientific reference regarding the possibility to expand connective tissue while maintaining its viability for subepithelial connective tissue grafting as well as reducing the size of the donor area and, consequently, postoperative morbidity and damage to important anatomical structures. Besides promoting the expansion of connective tissue, the tissue expansion device also produces grafts with uniform thickness. This allows a better adaptation of the graft to the recipient bed, resulting in a thinner clot layer, which decreases the chances of failure and tissue shrinkage since the clot layer acts as a barrier to plasmatic circulation, preventing adequate nourishment of the graft during the first stages of healing.[16] Another positive aspect is that smaller thickness facilitates the nourishment of the most internal layers of the graft and promotes faster reestablishment and maturation of the vascular plexus.[18] However, when the objective is to increase the thickness of the mucosa, this technique must be used with caution because the expansion device increases the area while decreasing the thickness of the graft, resulting in a graft that may not have the desired properties.

Since no histologic changes were observed within 60 days after surgery, it is possible to assert that the compression exerted by the device does not cause changes in the tissue that could compromise the use of this technique. In a prior study, samples of connective tissue were examined immediately after expansion, and the results revealed that the pressure exerted by the expansion device on the masticatory mucosa was not sufficient to rupture blood vessels, cells, or the collagen matrix.[19] These are very important results, because the collagen matrix determines differentiation and cell proliferation.[20] 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. Moreover, the integrity of the collagen structure is as important as the presence of viable cells for the success of the technique.[21]

The capacity of the connective tissue graft to induce epithelial differentiation was not the focus of this study, but since there was no coronal displacement of the graft, it was assumed that the newly formed tissue covering the recession was directly related to the implanted tissue and adequate nourishment of the recipient bed adjacent to the recession.

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 keratin layer are all factors that determine the maturity of the gingival tissue formed after grafting.[18] These determining factors were found in both test and control groups, which shows that

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the mechanical expansion of tissues does not cause significant biologic damage.

Conclusion

The results presented here indicate that this new technique to expand the area of connective tissue grafts is not only viable biologically, but also decreases surgery risks and postoperative morbidity without increasing costs and processing time.

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