치의과학 박사 학위논문

Gingival biotype modification with collagen matrix or autogenous subepithelial connective tissue graft – Histologic and volumetric analysis in dogs –

콜라겐 매트릭스와 상피하 결합조직을 이용한 치은 생체형 개선에 관한 연구 – 성견에서의 부피계측 및 조직학적 분석 –

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이 논문을 치의과학 박사 학위논문으로 제출함

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Abstract

Gingival biotype modification with collagen matrix or autogenous subepithelial connective tissue graft

– Histologic and volumetric analysis in dogs –

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1. Background: Gingival biotype modification (GBM) is a surgical procedure to increase the gingival thickness at sites where gingival recession could be expected such as anterior teeth. Although it is common to use subepithelial connective tissue grafts (SCTGs) from the palate of patients, several biomaterials are used with the development of material science and convenience of surgery. In this study, we evaluated the volumetric effect and biocompatibility of porcine type I collagen matrix graft (CG) on the GBM compared to SCTG in the beagle model.

2. Methods: Six adult dogs were used in this experiment. Each dog received an autologous SCTG from their palatal donor site or CG at the labial attached gingiva by sub-periosteal tunneling technique. Scanning dental stone model and three-dimensional digital volume analysis were conducted to compare the effects of CG and SCTG on gingival thickness increase before and after surgery. Histological and histomorphometric analysis were performed to evaluate the healing

pattern and biocompatibility of CG on the GBM at five months after the surgery.

3. Results: In the volume analysis, the increase of soft tissue thickness was 0.45 ± 1.07 mm and 0.69 ± 0.81 mm after one month, 0.06 ± 0.36 mm and 0.11 ± 0.32 mm after five months in the SCTG and CG groups, respectively with no significant difference between the groups. In the histological and histomorphometric analysis, the average soft tissue thickness was 1.80 ± 0.34 mm and 1.79 ± 0.40 mm in the SCTG and CG, respectively with no significant difference between the groups. Expressions of type I collagen and VEGF were found in both SCTG and CG groups at five months with no significant difference between groups in quantitative analysis.

4. Conclusions: CG and SCTG seem to have equivalent efficacy on the GBM in the volume increase and biocompatibility.

Keywords : collagen, connective tissue, gingival biotype, gingival recession, vascular endothelial growth factor
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1. Introduction

1.1. Study Background

Gingiva is the part of oral mucosa which covers the alveolar bone and surrounds the cervical region of teeth. If alveolar bone is lost for various reasons, the gingiva is also lost along with the bone, exposing the dental root, which can be esthetically and functionally disadvantageous[1]. Generally, gingival morphology has been described as 'scalloped and thin' or 'flat and thick' based on the thickness of the gingiva [2]. The term 'gingival biotype' refers to the gingival thickness in the facio-palatal or -lingual dimension, on the other hand, 'periodontal biotype' refers to not only the gingival thickness, but also other features including tooth shape, gingival contour, alveolar bone morphotype, and amount of keratinized gingiva [3]. A delicate, thin biotype can be more easily injured than the thick biotype and is more likely to induce gingival recession. Some studies have reported that with a thin gingival thickness of 2.0 mm or less, the initial peri-implant bone loss is accompanied by securing biological width of the gingiva [4-8]. This early bone loss can lead to gingival recession, which can be particularly problematic in the anterior maxilla, where esthetic results are essential [9]. Therefore, it is recognized that soft tissue with a thickness of 2.0 mm or more around implants play vital roles in preserving healthy peri-implant tissues and minimizing alveolar bone loss through biological protection [10]. In addition, alveolar bone dehiscence that arises during orthodontic treatment processes is proposed to cause gingival

recession [11]. Moreover, the possibility of gingival recession with orthodontic movement to the labial side through the cortical bone plate, further leading to alveolar bone dehiscence and soft tissue volume reduction, has been reported as a characteristic [12]. Hence, thin biotype gingiva can have a destructive effect on plaque-related inflammatory lesions, making it prone to tissue destruction [13], and is considered an important factor in gingival recession when it is associated with orthodontic treatment [14], meaning that the thickness of the gingiva not only has an important influence on the outcome of the root coverage procedure [15, 16] but also on orthodontic treatment. Therefore, procedures that increase the thickness of the gingiva should be considered before orthodontic treatment [17, 18].

Based on these, surgical procedure of gingival biotype modification (GBM) such as gingival tissue augmentation [5] has been proposed to increase the gingival thickness to maintain the gingival biologic width and minimize the loss of alveolar bone. In GBM, sub-epithelial connective tissue graft (SCTG) is commonly recognized as the ideal procedure [19, 20]. However, postoperative complications, such as uncontrolled bleeding, pain, and infection of the palatal donor site, limit its establishment as a routine procedure [21-24]. Collagen matrix graft (CG) is a material that can replace SCTG in gingival tissue augmentation, and several results similar to SCTG have been reported in increasing gingival thickness [25, 26].

1.2. Purpose of Research

In this study, we evaluated biocompatibility of CG and volumetric effects of CG and SCTG on GBM in the beagle model through three-dimensional (3D) digital volumetric and histomorphometric analysis.

2. Materials and Methods

2.1. Materials

A commercially available CG (Collagen Graft 2®, Genoss, Suwon, Korea) was used, which is a double-layered structure; upper layer is compact and lower layer is porous, and consisted of type I collagen from porcine tendon.

2.2. Experimental animals

Six adult beagle dogs (13 months and weight 13 kg) were employed. Sample size was determined based on the 3Rs principles in animal research. All dogs were housed in a cage under constant room temperature (22 ± 2℃) and humidity (50 ± 10%). The protocol was approved by the Institutional Animal Care and Use Committee, CRONEX, Seoul, Korea (approval No. 202003001) according to the ARRIVE guidelines for preclinical studies [27].

2.3. Study design

Twenty four sites of the labial side of upper and lower anterior teeth were included as recipient sites (four sites per animal) which were then randomly allocated into two groups; 1. SCTG and 2. CG. SCTG was obtained from the twelve donor sites of palatal vault. Each dog received an autologous SCTG (width x height x depth = 10.0 × 5.0 × 1.5–2.0 mm) from their palatal donor site or porcine type I CG (10.0 × 5.0 × 1.5–2.0 mm) at the labial side of the

second incisor. To evaluate and compare the effects of CG and SCTG on the increase in gingival thickness, dental cast models were fabricated with three time points, before the surgery and one and five months after the surgery, and 3D digital volumetric analysis was performed. After five months, all animals were sacrificed and samples were obtained for the histological, histometric, and immunohistochemical (IHC) analyses.

2.4. Surgical procedure

For the surgical procedure, all dogs were generally anesthetized with an intramuscular injection of zoletil (0.1 mg/kg; Zoletil®50, Virbac S.A, France) and xylazine hydrochloride (0.1 mg/kg; Rompun, Bayer, Germany) mixed in a 1: 1 ratio. Inhalation anesthesia was performed with 2% isoflurane (Isoflurane, Piramal Critical Care, United States) in 100% oxygen, and local infiltration anesthesia was done at donor and recipient sites with 2% lidocaine hydrochloride and 1: 100,000 epinephrine (Huons, Seongnam, Korea). Scaling and plaque control were conducted on all dogs before experimental surgery. At recipient sites, a vertical incision was made at the center of the second incisor and the third incisor on the buccal attached gingiva. Furthermore, a subperiosteal tunnel was formed mesially using a specifically designed elevator (CM9, Osung, Gimpo, Korea), allowing the graft to advance coronally to the marginal gingiva of the third incisor. At donor site, SCTG was harvested from both sides of the palatal vault and the adipose

tissue and epithelium were dissected from the graft (Figure. 1A). Hemostatic collagen matrix was applied to the donor site and a continuous locking suturing was conducted. The CG or SCTG was inserted into each subperiosteal tunnel (Figure. 1B) and an interrupted suture was conducted (Figure. 1C). After the surgery, subcutaneous administration of analgesic (carprofen, 5 mg/kg) and antibiotics (enrofloxacine, 0.2 ml/kg) was done for three days for pain relief and prevention of infection. The surgical sites were treated with 0.2% Chlorhexidine (Hexamedine, Bukwang Pharmaceutical, Seoul, Korea) daily for 10 days after the surgery.

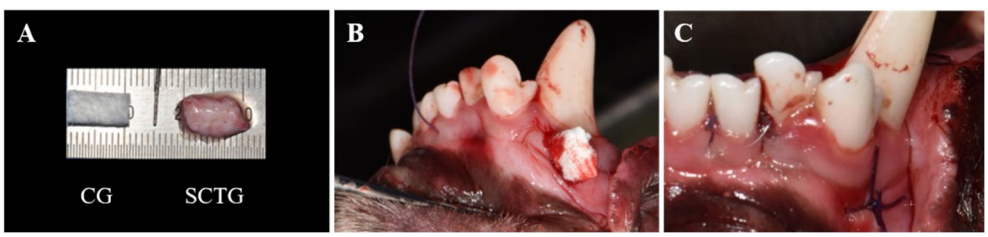

Figure 1. (A-C) Photographs of the surgical procedure. (A) Sizes of both matched CG (left) and SCTG from the palatal donor site (right) (10 × 5 × 1.5–2 mm). (B) Insertion of CG into the subperiosteal tunnel. (C) Closing vertical incision using the interrupted suture

2.5. Histometric analyses

After five months of surgery, euthanasia was conducted on all dogs using suxamethonium chloride hydrate (50 mg; Succipharm®, Komipharm, Gyeonggi, Korea). The resected specimens were fixed in a 10% neutral

buffered formalin. After dehydration and embedding in paraffin, 5-μm thick serial sections were performed. Three of the most central sections were selected; one stained with hematoxylin-eosin for histomorphometry and two for IHC. Microscopic examination and histomorphometric analysis were performed by two experienced researchers. Histometric measurement was performed with an image analysis software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Within the two imaginary lines drawn perpendicular to the second incisor axis at the base of the junctional epithelium (Line 1, Figure 2A) and the mucogingival junction (Line 4, Figure 2A), quaternary lines (Line 2 and 3, Figure 2A) were drawn within the soft tissue range. Then, the thickness of soft tissue, including the periodontal ligament, connective tissue, and epithelial tissue, was measured at quaternary lines, and statistical analysis (ANOVA, Kruskal–Wallis test) was conducted to analyze differences in the soft tissue thickness for each group.

2.6. Immunohistochemical analysis

The section was deparaffinized and hydrated, then antigen retrieval was performed with antigen retrieval buffer (Dako co., Glostrup, Denmark). Each section was incubated with the primary antibodies, anti-VEGF (Novus, nb100-664) and anti-Collagen I alpha (Novus, nbp1-77457) at room temperature for 1 hr, then secondary antibody (REAL Envision HRP Rabbit/Mouse Detection System, Dako co., Glostrup, Denmark) for 30 min.

The sections were assessed using a digital slide scanner and computer software (PANNORAMIC 250 Flash III and Caseviewer, 3DHISTECH Ltd. H-1141 Budapest, Öv u. 3., Hungary). For the IHC analysis, after drawing an imaginary line perpendicular to the second incisor axis at the base of the junctional epithelium and the mucogingival junction (MGJ), a 500 × 500 μm square adjacent to the root surface was set as the region of interest (ROI) (Figure 2B). Subsequently, color thresholds were set using an image analysis program (Image J, National Institutes of Health, US) for the regions where Col I and VEGF was observed and the expression of Col I and VEGF were quantified as an area ratio for each group.

2.7. Three-Dimensional digital volumetric analysis

Impressions were conducted before surgery, then one month and five months after surgery using a polyvinyl siloxane impression material (Aquasil Ultra LV®, Dentsply, Konstanz, Germany) and a tray, suitable for the oral structure of an adult dog fabricated with a 3D scanner and printer. After fabricating study casts using a super hard dental stone (SNOW ROCK®, Bluewin, Gunpo, Korea), casts were scanned using a dental scanner (ZEISS COMET 5M, Oberkochen, Germany). Then, image data were superimposed on the basis of the period (before, one month, and five months after the surgery) using software (Geomagic Design X and Control X, 3DSYSTEMS, SC, USA), with the 1st incisor as the reference point. Finally, 3D digital

volumetric analysis was conducted for the change of gingival thickness and volume. For the evaluation of gingival thickness change, a linear measurement using a cross-section method was performed [28] [29]. Imaginary lines on the second incisor connecting the most central gingival margins of the buccal and lingual side were set as a reference line on the cross-sectionally superimposed images based on period. Next, an A-line, which is perpendicular to the tooth axis was drawn to the reference line at a 2 mm apical point (Figure 2C). Then, changes of length in the A-line were evaluated on the basis of three time points; before surgery, one month, and five months after the surgery. For the evaluation of volumetric change, a measurement of the superimposed images of three time points was conducted using software (Geomagic Design X and Control X, 3DSYSTEMS, SC, USA). A rectangular area of 2.5 × 1.5 mm on the labial attached gingiva of the second incisor was set as the ROI and amount of change between periods was calculated (Figure 2D)[30].

2.8. Statistical analysis

Statistical analysis (ANOVA, Kruskal-Wallis test) was performed to analyze a difference in the soft tissue thickness for each group. Additionally, the independent sample *t*-test and Mann–Whitney *U* test compared the volume change between SCTG and CG. Then, the paired sample *t*-test and Wilcoxon signed-rank test were used for comparison based on period.

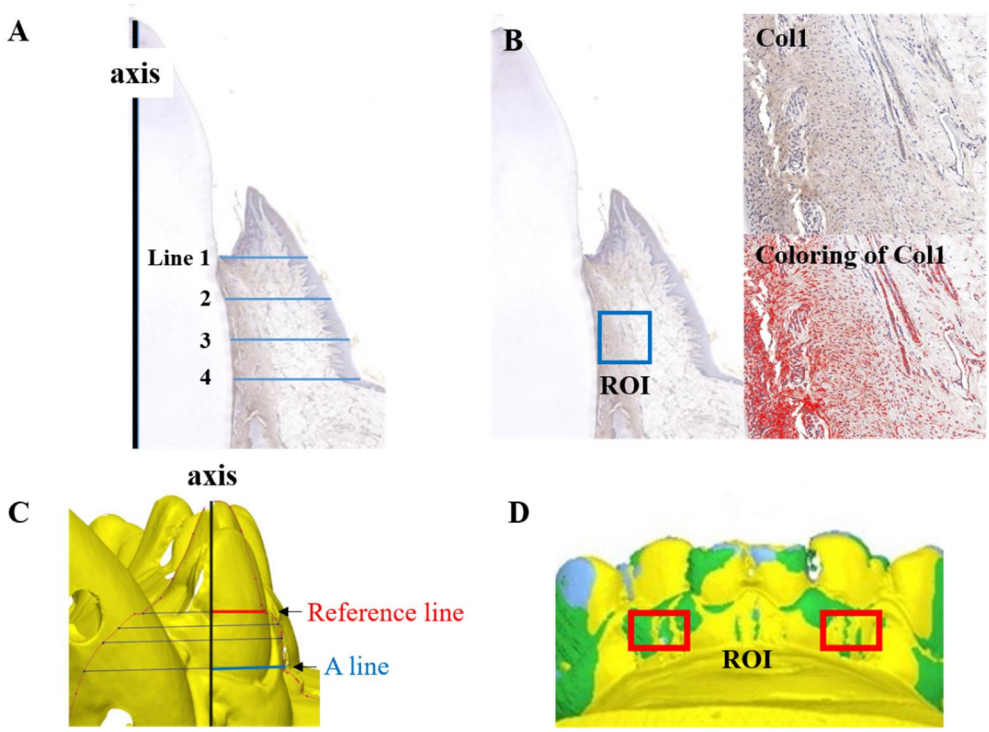

Figure 2. (A) An exemplary image showing histometric analysis of gingival thickness (H&E, type I collagen immune staining). Black line indicates tooth axis, and blue lines indicate imaginary lines perpendicular to tooth axis, where the measurement of the soft tissue thickness was conducted. Within the two imaginary lines drawn perpendicular to the second incisor axis at the base of the junctional epithelium (Line 1) and the mucogingival junction (Line 4), quaternary lines (Line 2 and 3, Figure 2A) were drawn within the soft tissue range. (B) (left) An exemplary image showing histometric analysis of quantification for type I collagen and VEGF. Blue square indicates ROI (500 × 500 μm square) for evaluating type I collagen and VEGF expression (H&E, type I collagen immune staining). (right bottom) The red section indicates

where type I collagen staining is observed using color threshold by Image J (30× original magnification). (C) Evaluation of gingival thickness change on the cross-section of the second incisor (black line: tooth axis, red line: reference line connecting the labial and lingual marginal gingiva, blue line (A-line): 2 mm apical to the baseline) (D) Superimposition of scanned data based on period, to evaluate volume changes (yellow: before surgery, blue: one month after surgery, green: five months after the surgery, red box: ROI)

3. Results

3.1. Clinical findings

The postoperative healing process was uneventful and there was no inflammatory sign on the surgical site.

3.2. Histometric findings

Table 1 lists the histometric analysis results. The average increase of gingival thickness was 1.80 ± 0.34 and 1.79 ± 0.40 mm in the SCTG and CG, respectively (Table 1). The use of CG showed an increase similar to that of SCTG. There was no significant difference between the groups.

Table 1. Histological evaluation of gingival thickness (Mann–Whitney *U* test between the groups; ㎛, Mean±SD)

Group	Line 1	Line 2	Line 3	Line 4	Average of Line 1-4
SCTG	1.64±0.29	1.79±0.37	1.85±0.36	1.94±0.44	1.80±0.34
CG	1.68±0.28	1.79±0.37	1.84±0.47	1.87±0.53	1.79±0.40

SCTG, subepithelial connective tissue graft; CG, collagen graft

3.3. Immunohistochemical analysis findings

Expression patterns of anti-Collagen I alpha and anti-VEGF in tissue sections detected by IHC staining (Figure 3) and the expression level was quantified with the area ratio of ROI. The expression level of Col1 was 13.25 ± 4.15% and 13.54 ± 5.39% in SCTG and CG groups, respectively, with no

statistically significant difference (p = 0.90). The expression level of VEGF in SCTG and CG groups was 3.24 ± 6.50% and 2.87 ± 3.29%, respectively, with no statistically significant difference (p = 0.55).

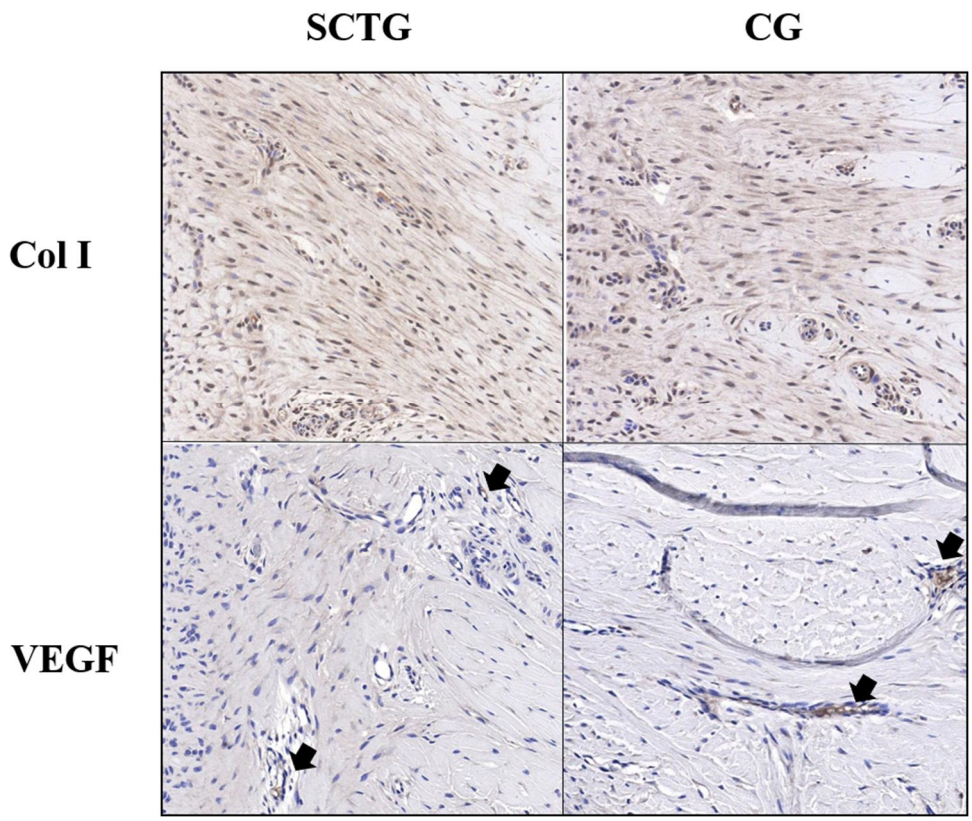

Figure 3. Representative images of the type I collagen (top row) and VEGF (bottom row) immunochemical staining in CG group (right column) and SCTG group (left column), respectively (30× original magnification). The black arrows indicate where VEGF staining was found.

3.4. Three-Dimensional digital analysis findings

The measurement of the change in gingival thickness and volume are

summarized in Table 2. In the 3D digital cross-section analysis of the dental cast model (Figure 2C), the increase of gingival thickness in soft tissue thickness after 1 month at the A-line was observed at 0.05 ± 0.45 mm and 0.07 ± 0.38 mm in the SCTG and CG groups, respectively. After five months, the increase in soft tissue thickness was 0.06 ± 0.36 mm and 0.11 ± 0.32 mm in the SCTG and CG groups, respectively with no significant difference was shown between the groups (Table 2).

In the 3D digital volumetric evaluation of the dental cast model (Figure 2D), an increase in soft tissue volume after one month was observed at 0.45 ± 1.07 mm^3 and 0.69 ± 0.81 mm^3 in the SCTG and CG groups, respectively. After five months, the increase in soft tissue volume was 0.48 ± 1.12 mm^3 and 0.70 ± 0.81 mm^3 in the SCTG and CG groups, respectively. The CG group at one month (p = 0.023) and five months (p = 0.035) showed a significant volume increase compare to volume at the baseline (Table 2).

Table 2. Change of gingival thickness and volume on the 3D digital evaluation (Wilcoxon-signed rank test for within group and Mann–Whitney *U* test between the groups; ㎛, Mean±SD)

	Group	Measurement	Baseline	Post op. 1 M	Post op. 5 M
Thickness (mm)	SCTG	A-line	3.27±0.62	3.32±0.46	3.33±0.43
		Change	0	0.05±0.45	0.06±0.36
	CG	A-line	3.19±0.62	3.26±0.36	3.30±0.44
		Change	0	0.07±0.38	0.11±0.32
Volume (mm^3)	SCTG	Volume	8.57±2.24	9.03±2.30	9.05±2.42
		Change	0	0.45±1.07	0.48±1.12
	CM	Volume	8.55±1.38	9.24±1.00*	9.25±1.43*
		Change	0	0.69±0.81	0.70±0.81

*: Significantly different from baseline ($p < 0.05$).

4. Discussion

This study evaluated and compared the effects of porcine type I collagen graft and SCTG on increasing gingival thickness using histomeric and 3D-digital analyses of dental casts. In 3D digital analysis, the CG group and SCTG group revealed an approximately 0.11 mm and 0.06 mm increase in gingival thickness on average at five months, respectively with no significant difference between the CG and SCTG groups. The histometric analysis also revealed results (CG: 0.18 mm, SCTG: 0.19 mm) similar to 3D digital analysis. These results support the previous studies that CG was not inferior to SCTG in gingival tissue augmentation [26, 29, 31, 32]. Alternatively, accurate and detailed value of increased gingival thickness is important in gingival augmentation surgery, not only for aesthetic improvement of the gingival depression, but also for the securement of gingival thickness of 2 mm or more around implants to prevent early bone loss in the process of securing the biological width of the gingiva [4-8]. Therefore, the effect of gingival augmentation surgery should be thoroughly reviewed [33].

In a dog experiment using a non-cross-linked CG and SCTG porcine, the maximum increase in gingival thickness after 10 months was 0.66 ± 0.29 mm in the SCTG group and 0.79 ± 0.37 mm in the CG group, respectively. However, an average increase in gingival thickness of 0.13 ± 0.26 mm in the CG group and 0.01 ± 0.26 mm in the SCTG group was reported [26]. An animal study in which immediate implant and soft tissue augmentation using cross-

linked CG was performed using the staged approach reported 0.52 mm increase in SCTG and 0.25 mm decrease in the CG group at the time point of sacrifice, and the author indicated that the underlying alveolar bone resorption offsets the increase in gingival volume [29]. Subsequently, a human study using cross-linked CG grafts was performed around implant sites and evaluated through 3D digital analysis three months after the operation. A 0.175 mm increase in the CG group and 0.51 mm in the SCTG group on crest were reported. The study also reported 0.59 mm for the CG group and 0.94 mm for the SCTG group on buccal ROI, with no significant difference between the groups [31]. Hence, judging from these results, it is considered that the effect of gingival augmentation in the dogs is inferior to that in humans because of the relatively thin gingival thickness (1.79 mm in this study, Table 1), narrow attached gingiva width of the dog, and behavioral control causing worse results in gingival augmentation [15, 16].

The type of CG used and post-operative period should also be considered. In a study where the biodegradation of both cross-linked and non-cross-linked membranes was evaluated, the cross-linked membrane showed the initiation of blood vessel invasion at eight weeks, while the entire organization and biodegradation were observed at 4 weeks in the non-cross-linked membrane [34]. Most studies on gingival tissue augmentation using non-cross linked CG reported shrinkage of the soft tissue volume from immediately after the procedure to one, three, and up to six months [29, 31, 35, 36]. However, this study showed no significant change in gingival thickness or volume from one

month to five months. It seems that the difference between these results depends on the type of collagen matrix used. In this study, cross-linked porcine matrix used was completely biodegraded and blended into the tissue at five months, as shown by the histological analysis. Thus, since no significant change in gingival thickness and volume from one month to five months was observed, findings propose that biodegradation of CG would have occurred within five months without volume change.

Type I collagen is predominant in the reparative connective tissues [37]. In this study, expression of type I collagen was found in both SCTG and CG groups with no significant difference between the groups in quantitative analysis, which implies that augmented gingival tissue with collagen substitutes is equivalent to that with SCTG in the aspect of tissue quality as reported in a previous study [38].

VEGF is the best known angiogenic factor and is up-regulated during early wound healing phase [39]. Although little is known about the association between soft tissue graft and the expression of VEGF, there are studies suggesting that VEGF may play an important role in vascularization in the engraftment process [38 40]. After SCTG harvesting from palatal donor site, free gingival tissues are separated from blood circulation, followed by necrotic process, which is a known stimulatory factor for expression of VEGF [41]. In this study, SCTG group and CG group both showed VEGF expression five months after the graft procedure, which implies collagen substitutes are engrafted through similar process to SCTG.

This study had several limitations. First, sacrifice was conducted five months after the gingival augmentation procedure. However, in order to obtain biological insight of the role of VEGF and type I collagen during the biodegradation and healing process of the recipient site, a multiple time points of sacrifice are needed, instead of one time point of sacrifice. Since the primary goal of this experiment was to compare the efficacy of CG and SCTG, it was inevitable to sacrifice a small number of animals at five months to evaluate tissue quality after stabilization of graft materials. Relatively few study animals which may influence statistical results were also limitation of the study. Finally, interpretation of the results in this study requires close attention because several factors previously mentioned (types of collagen matrix, postoperative period, difference between human and animal study, location –buccal or crest, etc) affects the results of the gingival augmentation.

5. Conclusion

Within the limitations of the study, sub-epithelial connective tissue graft (SCTG) and type I collagen graft (CG) seem to have equivalent efficacy in gingival thickness augmentation. Furthermore, recipient sites of both SCTG and CG showed similar histologic appearance in expression of type I collagen and VEGF, which implies newly formed tissues from both SCTG and CG graft are equivalent in quality.

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국 문 초 록

1. 연구배경: 치은 생체형 개선은 전치부 등 치은퇴축이 예상되는 부위에서 치은 두께의 증가를 위해 시행되는 술식이다. 치은 생체형 개선은 통상 환자의 구개측에서 채취한 상피하 결합조직(SCTG)이 사용되지만, 재료과학의 발전으로 콜라겐 매트릭스(CG) 등을 포함한 여러가지 대체재가 개발되어져 왔다. 본 연구에서는 비글견의 순측 부위에 콜라겐 매트릭스 이식편과 상피하 결합조직을 이식한 후, 연조직의 부피변화와 이식재료의 생체적합성을 비교 평가하였다.

2. 연구방법: 6마리 성견이 이 실험에 사용되었다. 각 성견의 전치부 순측 각화치은 부위에 각 성견의 전치부 순측 각화치은에 수직절개를 시행을 하고, 재료를 삽입하기 위한 골막하 낭(subperiosteal pouch)을 형성하여 구개측에서 채득한 상피하 결합조직(SCTG) 혹은 콜라겐 매트릭스 이식편(CG)을 이식하였다. 술전과 술후 1개월 및 5개월에 치과용 석고 모델을 스캔하여 3차원적 디지털 부피 계측을 진행하였다. 술후 5개월에 실험동물 희생 후, 조직학적 분석을 통해 치유양상과 생체적합성을 평가하였다.

3. 결과: 모든 군에서 임상적인 부작용은 없었다. 3차원적 디지털 부피 계측에서 1개월 후 연조직 두께는 SCTG군에서 0.45 ± 1.07 mm, CG군에서 0.69 ± 0.81 mm 증가하였고, 5개월 후 SCTG군에서 0.06 ± 0.36 mm, CG군에서 0.11 ± 0.32 mm 증가하였다. 두 군 간 통계적으로 유의한 차이는 관찰되지 않았다. 술후 5개월에 두 군에서 모두 제1형 콜라겐과 혈관내피성장인자가 관찰되었으며 발현 정도에 있어서 두 군 간 통계적으로 유의한 차이는 관찰되지 않았다.

(4) 결론: 이 연구에서 사용된 콜라겐 매트릭스는 구개부에서 채득한 결합조직과 비교하여 치은생체형 개선에 있어서 부피 변화와

생체적합성에서 유의한 차이가 없는 것으로 보인다.

주요어 : 결합조직, 치은생체형, 치은퇴축, 콜라겐, 혈관내피성장인자
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