Oral Soft Tissue Regeneration Using Nano Controlled System Inducing Sequential Release of Trichloroacetic Acid and Epidermal Growth Factor

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Abstract

BACKGROUND: The effect of nano controlled sequential release of trichloroacetic acid (TCA) and epidermal growth factor (EGF) on the oral soft tissue regeneration was determined.

METHODS: Hydrophobically modified glycol chitosan (HGC) nano controlled system was developed for the sequential release of TCA and EGF, and the release pattern was identified. The HGC-based nano controlled release system was injected into the critical-sized defects created in beagles' palatal soft tissues. The palatal impression and its scanned body was obtained on various time points post-injection, and the volumetric amount of soft tissue regeneration was compared among the three groups: CON (natural regeneration control group), EXP1 (TCA-loaded nano controlled release system group), EXP2 (TCA and EGF individually loaded nano controlled release system). DNA microarray analysis was performed and various soft tissue regeneration parameters in histopathological specimens were measured.

RESULTS: TCA release was highest at Day 1 whereas EGF release was highest at Day 2 and remained high until Day 3. In the volumetric measurements of impression body scans, no significant difference in soft tissue regeneration between the three groups was shown in two-way ANOVA. However, in the one-way ANOVA at Day 14, EXP2 showed a significant increase in soft tissue regeneration compared to CON. High correlation was determined between the histopathological results of each group. DNA microarray showed up-regulation of various genes and related cell signaling pathways in EXP2 compared to CON.

CONCLUSION: HGC-based nano controlled release system for sequential release of TCA and EGF can promote regeneration of oral soft tissue defects.

Kwang Man Park and Hong Jae Lee have contributed equally to this work.

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Keywords Trichloroacetic acid · Epidermal growth factor · Nano controlled release system · Soft tissue regeneration · Canine palate · Human gingival fibroblast

1 Introduction

Gingival recession is a downward movement of marginal gingiva toward cemento-enamel junction, causing exposure of dental root [1]. In the occurrence of gingival recession in the anterior teeth, esthetic problems and oral exposure of the root can lead to problems such as dentin hypersensitivity and plaque accumulation [2]. In 1985, Miller classified gingival recession into four categories according to the pattern of gingival recession, and emphasized the degree of loss of alveolar bone or soft tissue as an important variable that affected the treatment results. Also Miller reported if the gingival recession progressed to the cemento-enamel junction and under, treatment results would be compromised [3]. Treatment methods for gingival recession include pedicle flap technique, free gingival graft, guided tissue regeneration and connective tissue graft. The success rate of these methods was reported to be 80–88% on average [4], but it is highly difficult to regenerate the missing papilla in such occasions of class III and class IV of the miller's classification.

In dermatology, chemical regeneration of skin scars (CROSS) method using trichloroacetic acid (TCA) has been used to induce skin tissue regeneration. Skin application of TCA causes collagen necrosis in the papillary region above the reticular dermis, followed by the coagulative necrosis and protein precipitation of cells in the epidermis. During the first few days after TCA application to the skin, the necrotic layer is peeled off and re-epithelialization proceeds, which causes the skin to thicken due to the reconstitution of its components and the increase in collagen content and volume. The use of TCA in CROSS method has been clinically proven for skin cosmetics in a wide range of areas including wider hair follicle, acne scars, solar lentigo, melasma and freckles [5].

Unlike dermatology, the use of TCA in dentistry is still limited in the treatment of oral soft tissue. Among the literature reporting the use of TCA for the treatment of oral soft tissue, the application 30% TCA in oral retinoid-induced cheilitis and pemphigus vulgaris was reported to be

highly effective [6, 7]. However, the simple local application of TCA for regeneration of recessed gingiva comprehends problems that are more dangerous than skin application, which makes it impossible to induce slow penetration of strong TCA through the oral keratinized epithelium to the connective tissue layer. In order to solve this problem, it is necessary to inject the TCA directly into the epithelium-connective tissue boundary layer, and to control the slow release at a low concentration [8].

The drug delivery system is a technique to control the release rate of a drug and efficiently deliver it to a target site, maximizing efficacy while minimizing the side effects. Drug delivery systems using small size nanoparticles have unique physical, chemical and biological properties, such as allowing them to traverse cell and tissue barriers, but also improves drug responsiveness [9]. Chitosan is obtained by alkaline deacetylation of chitin, which is a rich polysaccharide next to cellulose [10]. Chitosan is a highly utilized substance due to its superior biocompatibility, biodegradability, high charge density, mucous membrane adhesion and low toxicity. However, chitosan was limited in its use because of its insolubility in a biological solution at pH 7.4. Thus, the manufacture of hydrophobically modified glycol chitosan (HGC) nanoparticles made of hydrophilic glycol chitosan conjugated with hydrophobic β -cholanolic acid was reported [11]. The HGC nanoparticles are self-aggregated in aqueous solution, encapsulates various chemicals and peptides, are continuously released, and are well soluble in a neutral pH solution [12].

In spite of the tissue necrotic nature of TCA, gene expression analysis of human gingival fibroblasts cultured in TCA-contained media were confirmed to show up-regulation of growth and activity-related genes such as the epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) in our preliminary study. In addition, more than 400 significantly up-regulated genes were verified to up-regulate various cell signaling pathways associated with cell growth, regeneration and proliferation [8]. Encouraged from our preliminary study results and in order to apply the unique concept of CROSS, which sequentially induces tissue necrosis, healing and regeneration in skin, to mucosal-based oral soft tissue regeneration, we have developed the HGC based nano controlled release system for inducing controlled sequential release of TCA and EGF. The safety and effectiveness of the TCA- and EGF-leaded HGC were evaluated in cell- and animal models, followed by laying the foundation for the development of new nanotechnology-based therapies for oral soft tissue regeneration.

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2 Materials and methods

2.1 Reagent

Glycol chitosan (MW = 250,000 g/mol, degree of polymerization ≥ 400), 5 β -cholanolic acid, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl), TCA (Sigma-Aldrich Co., St. Louis, MO, USA), methanol (Sigma-Aldrich Co., St. Louis, MO, USA), and epidermal growth factor (Peprotech, London, UK) were purchased and utilized.

2.2 Fabrication of 5 β -cholanolic acid-conjugated glycol chitosan nanoparticles

The conjugation of 5 β -cholanolic acid to glycol chitosan was performed based on a literature procedure [11]. Recently, we fabricated 5 β -cholanolic acid-conjugated glycol chitosan by a modification process [13]. In brief, glycol chitosan (1.0 g, 0.4×10^{-5} mol) and 5 β -cholanolic acid (123.0 mg, 0.34×10^{-3} mol) were dissolved in 10 ml of distilled water/methanol (1:1, v/v), followed by adding *N*-hydroxysuccinimide (58.6 mg, 0.51×10^{-3} mol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (97.6 mg, 0.51×10^{-3} mol) into the mixture, and the conjugation reaction was induced at room temperature for 24 h. After the reaction, dialysis was performed using a dialysis membrane (molecular weight cut-off (MWCO): 3500 g/mol) for 24 h for purification. The dialysis solvent was the mixture of distilled water and methanol. Then, the resultant was lyophilized to obtain final HGC nanoparticles.

2.3 Manufacture of TCA-loaded nanoparticles

To prepare TCA-loaded nanoparticles, 10 mg of HGC was dispersed in 10 ml of distilled water, and TCA (1.0 mg, 0.6×10^{-5} mol) was dissolved in 0.025 ml of distilled water to prepare a solution. Thereafter, the TCA solution was added to the HGC solution, and then cross-reacted at 600 rpm at room temperature. After 24 h, dialysis was performed for 24 h using a dialysis membrane (MWCO: 3500 g/mol) to remove unloaded TCA, and the final TCA-loaded nanoparticles were obtained through lyophilization. The amount of TCA-loaded was confirmed by quantitative analysis of Cl using ion chromatography. To manufacture 0.5% TCA-loaded nanoparticle solution, 4 ml of distilled water was added to TCA-loaded nanoparticles (2.8 mg) (0.00714 mg TCA/1 mg TCA-loaded nanoparticles).

2.4 Manufacture of EGF-loaded nanoparticles

To manufacture the EGF-loaded nanoparticles, 10 mg of HGC was dispersed in 10 ml of distilled water, and 0.1 mg of EGF (0.1 mg, 0.15×10^{-7} mol) was dissolved in 0.1 ml of distilled water to prepare a solution. Thereafter, the EGF solution was added to the HGC solution, and cross-reacted at 300 rpm at room temperature. After 24 h, dialyzing was carried out for 24 h using a dialysis membrane (MWCO: 10,000 g/mol) to remove the unloaded EGF, and the final EGF-loaded nanoparticles were obtained through lyophilization. The amount of EGF-loaded was confirmed by ELISA (Human EGF standard ABTS ELISA development kit, Peprotech, London, UK). To prepare a complex solution of 0.5% TCA-loaded nanoparticles and 80 μ g EGF-loaded nanoparticles, 2 ml of distilled water added to TCA-loaded nanoparticles (2.8 mg) and 2 ml of medium added to a solution of EGF-loaded nanoparticles (8.9 mg) were mixed (8.98 μ g EGF/1 mg EGF-loaded nanoparticles).

2.5 Evaluation of nano controlled release system

The release patterns of TCA and EGF from HGC nanoparticles were investigated in pH 7.4 phosphate buffered solution (PBS, Gibco BRL, Grand Island, NY, USA). The nanoparticle immobilization was performed using the concentration of 2 g/l nanoparticles. The immobilized nanoparticles were immersed in 3 ml PBS and shaken at 150 rpm and 37 °C incubator. The release medium was completely replaced at each predetermined time interval and all samples were stored at 20 °C until they were inspected. TCA and EGF released from the supernatant were analyzed by ELISA development kit (Peprotech, London, UK) according to the manufacturer's protocol.

2.6 Cell culture

Human gingival fibroblasts were purchased from ATCC (ATCC[®] PCS-201-018TM, Manassas, VA, USA). Cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corporation, Carlsbad, CA, USA) containing antibiotics and 10% fetal bovine serum (FBS, Sigma-Aldrich Co., St. Louis, MO, USA). The suspended human gingival fibroblasts were cultured in humidified incubator at 95% air, 5% CO₂ and 37 °C. Cultured human gingival fibroblasts (2–3 weeks) were used in this study.

2.7 Cytotoxicity assessment

Cytotoxicity was assessed 16 h after application of 0.5% TCA, as determined by previous preliminary studies. Besides the control group, 0.5% TCA, and 0.5% TCA-

loaded nanoparticle were applied to cultured human gingival fibroblasts, and cell proliferation was analyzed using the cell counting kit (CCK-8, Dojindo, Kumamoto, Japan). The reaction products were transferred to 96-well plates and read at 450 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

2.8 RT-PCR

RT-PCR was performed in the control-, 0.5% TCA-, and 0.5% TCA-loaded nanoparticle group with cultured human gingival fibroblasts. Differences in gene expression were checked between 0 (immediately after application), 3, 9 and 21 h, respectively. Total RNA was extracted using TRIZOL lysis solution (TRIZOL[®] Reagent, GIBCO BRL, Carlsbad, CA, USA), and concentration was performed after probe labeling. To identify changes in gene expression level, the oligonucleotide primer sequences of the growth factors were confirmed (Supplementary Table 1). The synthesized cDNA samples were heated at 94 °C for 5 min and amplified at 94 °C for 30 s, 59 °C for 45 s, and 72 °C for 30 s for 35 cycles. PCR products were extracted on 2% agarose gel using ethidium bromide-containing Tris–acetate–EDTA buffer (Tris–acetate–EDTA buffer, Sigma-Aldrich Co., St Louis, MO, USA), and separated under ultraviolet light.

2.9 Adult canine experiment

For the experiment, animals were grouped into three: natural regeneration group (control group, CON), TCA-loaded nano controlled release system group (experimental group 1, EXP1), and TCA and EGF individually loaded nano controlled release system group (experimental group 2, EXP2).

2.10 Measuring defect volume using impression body scan and histopathological specimen

One-year old, 10 kg beagles (N = 10) were used with the approval of institutional animal care and use committee (CRONEX-IACUC 201803002) for the prior clinical confirmation of the biological potential of TCA for the healing of oral mucosal wound, which was carried out in compliance with the ARRIVE guidelines. All surgery and follow-up procedures were performed under general anesthesia, using tiletamine/zolazepam (0.1 mg/kg, ZOLETIL[®] 100, VIRBAC FRANCE, Carros, France), xylazine (2.3 mg/kg, Rompun Inj., BAYER KOREA, Seoul, Korea), atropine sulfate (0.05 mg/kg, Atropine sulfate Inj. 10 mg, JE IL PHARMACEUTICAL, Daegu, Korea). After the surgery, analgesics (Zipan Inj. 100 mg, ILSUNG Pharmaceuticals, Seoul, Korea) was administered for 3 days. For this study,

the defect model of palatal mucosa described in the literature was applied [14, 15]. The surgical procedure was as follows. The palate mucosa was removed using a biopsy punch to form a standardized 6 mm diameter cylindrical defect in palates of 10 beagles. In order to form six soft tissue defects in each beagle, three defects were symmetrically positioned with respect to the palatal midline (Fig. 1A). Treatment of palatal defect and follow up are as follows. One day after surgery, the beagles were randomly selected as described below. For the formation of CON (N = 20), EXP1 (N = 20) and EXP2 (N = 20), two of the six defects of each beagle were randomly selected. For each defect, a disposable syringe (Kovax-Syringe[®] 1 mL, Korea Vaccine Co. LTD, Ansan, Gyeonggi, Korea) was used for injecting the nano controlled release system (Fig. 1B). To measure the volume of palatal tissue damage, customized individual impression trays were fabricated and impression (Examixfine Regular, GC Co., Tokyo, Japan) of the palates were taken on days 1, 3, 7, 14 (Fig. 1C, D). Each impression body was scanned (DOF, Swing, Seoul, Korea) to compare volume change using computer software (3D Systems, Geomagic Design X, Rock Hill, SC, USA & Cimatron, Elite, Rock Hill, SC, USA) (Supplementary Fig. 1). In order to measure histopathological specimens, beagles were sacrificed on Day 7 and 14, and the parameters such as new epithelium length, area, width, and thickness were measured on the soft tissue regeneration in histopathological specimens stained with H&E (hematoxylin and eosin) and Masson's Trichrome (Supplementary Fig. 2).

2.11 DNA microarray and KEGG pathway analysis

The up-regulated or down-regulated gene by the nano controlled release system were confirmed. On Day 3, beagles were sacrificed and DNA microarray analysis using the harvested palatal tissues from CON and EXP2 were performed. Total RNA was extracted using TRIZOL lysis solution (TRIZOL[®] Reagent, GIBCO BRL, Carlsbad, CA, USA), followed by the concentration after probe labelling. Affymetrix gene chip was used and images were extracted with a genechip scanner (Affymetrix GeneChip[®] Scanner 3000 7G, Affymetrix, Santa Clara, CA, USA). Data were extracted using a dedicated software (Affymetrix GeneChip[®] Command Console[®], Affymetrix, Santa Clara, CA, USA) and data were standardized using a standardization program (Expression Console 1.4, Affymetrix, Santa Clara, CA, USA). Genes showing significant differences in expression by more than 1.5-fold were identified through comparison of control and experimental groups with the differentially expressed gene (DEG) finding. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis was used to map the expression of the DNA

Fig. 1 Soft tissue defect formation, injection, and impression on the soft tissue of beagle's palate. **A** A biopsy punch was used to remove full thickness palatal mucosa for making a standardized 6-mm diameter cylindrical defect. The defects were symmetrically located with respect to the palatal midline to form six soft tissue defects. **B** Injections to area of soft tissue defects. **C** Impression taking of palate defects. **D** Impression body. yellow scale bar: 6 mm

chip, and the change in gene expression level was determined at the pathway level according to experimental conditions.

2.12 Statistical analysis

One-way ANOVA was used for cytotoxicity test, one-way and two-way ANOVA was used for measurement of soft tissue defect volume by scans, Kruskal–Wallis analysis was used for measurement of histopathologic specimens, and Pearson's correlation analysis was used for comparative analysis of scan value and histopathological specimen measurements ($p < 0.05$, SPSS 18.0, SPSS Inc, Chicago, IL, USA).

3 Results

3.1 Development of nano controlled release system

HGC nanoparticles self-aggregate in aqueous solution, encapsulating various chemicals and peptides, characterized by sustained release and good solubility in neutral pH solution. Chitosan, used for manufacturing HGC-based nano controlled release system, has the hydrophilic cationic macromolecule amine (NH_2) which is conjugated with

hydrophobic cholanolic acid. Hydrophobic cholanolic acid forms the inner core by self-assembly in water, and hydrophilic cationic chitosan forms the outer shell (Fig. 2). Anionic TCA and EGF were then loaded onto the chitosan shell by ionic bonding, and their release patterns were controlled (Fig. 3).

3.2 Release of TCA and EGF by nano controlled release systems

TCA and EGF release patterns by HGC-based nano controlled release system over time were evaluated. The maximum amount of TCA released on Day 1 showed a sharp decline, and the EGF release peaked at 2 days, then remained high until 3 days and then decreased rapidly (Fig. 4).

3.3 Cytotoxicity assessment

0.5% TCA and 0.5% TCA-loaded nanoparticle were applied in human gingival fibroblasts, and cytotoxicity was assessed 16 h later using a cell counting kit. As a result, the highest cell proliferation was observed in CON than other groups using TCA, suggesting that experimental groups of both 0.5% TCA and 0.5% TCA-loaded nanoparticle

Fig. 2 Fabrication of the Hydrophobically modified glycol chitosan (HGC)-based nano controlled release system. Chitosan, which has a hydrophilic cationic polymer– amine group (NH₂), was conjugated

with hydrophobic cholic acid to prepare nanoparticles. Hydrophobic cholic acid forms core by self-assembly in water, and hydrophilic cationic chitosan forms shell

Fig. 3 Anionic TCA and EGF were loaded to chitosan shell by ionic bond, respectively

showed statistically significant increase in cytotoxicity compared to CON (Fig. 5).

3.4 RT-PCR

For RT-PCR using human gingival fibroblasts, 0.5% TCA and 0.5% TCA-loaded nanoparticle were compared for growth factor gene expression at 0, 3, 9 and 21 h. As the loading control, β-actin was used to confirm the significance of the experiment. Similar to 0.5% TCA group, 0.5% TCA-loaded nanoparticle upregulates cell expression of EGF, EGFR, fibroblast growth factor-1 (FGF-1), fibroblast growth factor-2 (FGF-2), keratinocyte growth factor (KGF), Platelet derived growth factor-β (PDGF-β), transforming growth factor-α (TGF-α), transforming growth

Fig. 4 The release pattern induced by the HGC-based nano controlled release system. Note that TCA decreased rapidly after maximal release on Day 1, and EGF remained high until Day 3 and then decrease rapidly. Red square: EGF. Blue circle: TCA

Fig. 5 Cytotoxicity test results of TCA determined by the cell proliferation assay after 16 h incubation for natural regeneration group (control group, CON), TCA injection group (TCA), and TCA-loaded nano controlled release system group (TCA + Nano). **Significant differences were noted between CON and TCA or TCA-loaded nanoparticle ($p < 0.01$)

factor- β 1 (TGF- β 1) and vascular endothelial growth factor (VEGF) (Fig. 6).

3.5 Measuring defect volume using impression body scan and histopathological specimen

Among ten beagles, eight remained healthy until the sacrifice. Two beagles were replaced due to expiration, on the Days 3 and 7 respectively, during the experiment under general anesthesia. Since the impression bodies were scanned to measure tissue defects, the volumetric measurement of lower values compared to the higher values were considered as the result of the relatively greater amount of newly regenerated soft tissues. The overall volume in all three groups of CON, EXP1, and EXP2 increased up to Day 3 and decreased thereafter. There was no significant difference in soft tissue regeneration between the three groups via two-way ANOVA (Fig. 7A). However, from the one-way ANOVA using only the results of Day 14, EXP2 showed significant decrease in volume, suggesting significant increase in soft tissue regeneration compared to CON (Fig. 7B).

On Day 7 and 14, four parameters such as the new epithelium length, area, width, and thickness were measured on the soft tissue regeneration site in histopathology specimen under staining with H&E and Masson's Trichrome. However, no significant differences were noted between the three groups concerning any of the four

parameters measured (data not shown). From the Pearson's correlation analysis result, high correlations between the results of each group were verified. In particular, at Day 14, there was a high correlation up to -0.961 of the correlation coefficient value between scanned volume and thickness values (Table 1).

3.6 DNA microarray and KEGG pathway analysis

On Day 3, beagles were sacrificed and the DNA microarray analysis of the palatal tissues from CON and EXP2 were performed in order to investigate the difference in gene expression of *canis familiaris* when HGC-based nano controlled release system was applied. The affymetrix gene chip was used to identify 27681 genes, and 567 genes with a change greater than 1.5-fold ($p < 0.05$) were determined. KEGG pathway analysis was performed on 567 genes in order to identify the relevant cell signaling pathways, and 27 pathways involved in cell growth, regeneration, and proliferation were identified.

In ribosome, MRPL12, MRPL13, MRPL17, MRPL27, MRPL36, RPL15, RPL18A, RPL22, RPL24, RPL27, RPL28, RPL29, RPL3, RPL30, RPL35, RPL37A, RPL4, RPL5, RPL6, RPL9, RPLP0, RPS11, RPS13, RPS15, RPS16, RPS17, RPS18, RPS19, RPS2, RPS21, RPS23, RPS25, RPS27A, RPS3, RPS4X, RPS5, RPSA were upregulated (Supplementary Fig. 3). In ribosome biogenesis in eukaryotes, FBL, GAR1, GTPBP4, NOP56, NOP58, RAN, ROK2, WDR3, WDR36, WDR43 and XRN2 were upregulated (Supplementary Fig. 4). In pathway in cancer, CALML5, CCDC6, CXCL12, DAPK2, EDNRA, FGF7, FGFR1, FGFR2, FZD6, GNG12, GSTA4, HSP90AB1, JUP, KITLG, LAMA4, LAMB1, LAMC1, MGST3, MMP2 and PDGFRA were upregulated and SLC2A1 was downregulated (Supplementary Fig. 5). In PI3K-Akt signaling pathway, COL1A1, COL1A2, COL6A3, FGF7, FGFR1, FGFR2, GNG12, HSP90AB1, ITGB5, KITLG, LAMA4, LAMB1, LAMC1, PDGFRA, SPP1 and TNC were upregulated (Supplementary Fig. 6). In regulation of actin cytoskeleton, ARPC1A, CFL2, CXCL2, DIAPH3, FGF7, FGFR1, FGFR2, GNG12, ITGB5, NCKAP1, PDGFRA and PFN were upregulated (Supplementary Fig. 7). In focal adhesion, COL1A1, COL1A2, COL6A3, ITGB5, LAMA4, LAMB1, LAMC1, PDGFRA, SPP1 and TNC were upregulated (Supplementary Fig. 8). In ECM-receptor interaction, COL1A1, COL1A2, COL6A3, ITGB5, LAMA4, LAMB1, LAMC1, SPP1 and TNC were upregulated (Supplementary Fig. 9). In MAPK signaling pathway, FGF7, FGFR1, FGFR2, GNG12, HSPB1, KITLG, PDGFRA and STMN1 were upregulated (Supplementary Fig. 10). In cell cycle, ANAPC4, CDK1, MCM6, PTTG1 and SFN were upregulated (Supplementary Fig. 11). In EGFR tyrosine kinase inhibitor resistance,

Fig. 6 RT-PCR results of TCA injection group (TCA) and TCA-loaded nano controlled release system group (TCA + nano). Note that 0.5% TCA + nano induced up-regulation group of all tested genes similar to 0.5% TCA group

Fig. 7 Comparison result of the scanned impression volume of the canine palatal soft tissue defects between natural regeneration group (control group, CON), TCA-loaded nano controlled release system group (experimental group 1, EXP1), and TCA and EGF individually loaded nano controlled release system group (experimental group 2, EXP2). **A** Volume change over time. Note that volume increases up to

Day 3 and then decrease. From the two-way ANOVA, no significance differences were noted between groups. **B** Result of the comparison using one-way ANOVA between groups on Day 14. **EXP2 showed significant increase of soft tissue regeneration compared with CON ($p < 0.01$)

Table 1 Pearson's correlation analysis results between the parameters of the scanned impression volume of the canine palatal soft tissue defects (scan), new epithelium formation around the defects (new epithelium), defect area (area), defect width (width), and defect

Parameter	Day14 scan	Day14 new ep.	Day14 area	Day14 width
Day14 new epithelium	− 0.926**			
Day14 area	− 0.911**	0.928**		
Day14 width	− 0.933**	0.936**	0.920**	
Day14 thickness	− 0.961**	0.952**	0.939**	0.947**

**Correlation is significant ($p < 0.01$)

thickness (thickness) at Day 14 post-injection of the nano controlled system inducing sequential release of trichloroacetic acid and epithermal growth factor

FGFR2 and PDGFRA were upregulated (Supplementary Fig. 12). Gene expression was up-regulated in several other intracellular signaling pathways (Supplementary Table 2). Full names and accession numbers of the verified genes in *canis familiaris* from the DNA microarray analysis in this study are presented (Supplementary Table 3).

4 Discussion

We developed the HGC-based nano controlled release system in order to avoid the risk of simple TCA application on oral soft tissue. HGC-based nano controlled release system was used to inject TCA and EGF into the epithelial-connective tissue boundary layer. HGC nanoparticles composed of hydrophilic glycol chitosan conjugated with hydrophobic β -cholanolic acid are self-aggregating in aqueous solution, encapsulating various chemicals and peptides, sustained release, and are well soluble in neutral pH solutions. The HGC Based nano controlled release system was evaluated and observations were made first as to whether the release of EGF was sequential after TCA

release. The maximum amount of TCA released on Day 1 showed a sharp decline, and the EGF release peaked at Day 2, then remained high until Day 3 and then decreased rapidly. Cell cytotoxicity experiments were also performed using cell counting kits 16 h after application of TCA to human gingival fibroblasts. The highest cell proliferation was observed in the control without TCA than in 0.5% TCA and 0.5% TCA loaded nanoparticles, indicating that application of 0.5% TCA and 0.5% TCA loaded nanoparticles resulted in greater cytotoxicity than that of control. RT-PCR, in human gingival fibroblasts, was performed to evaluate whether or not the major growth factor genes would be upregulation in spite of greater cytotoxicity. The 0.5% TCA loaded nanoparticles upregulated most of the growth factors evaluated. Based on the results of the series of evaluations, it was confirmed that the HGC-based nano controlled release system can upregulate several growth factor genes in oral soft tissue cells by inducing sequential release of TCA and EGF.

In measurement of canine palatal soft tissue defect volume using impression body, there was no significant difference between the groups of CON, EXP1, and EXP2

in terms of two-way ANOVA. In the one-way ANOVA using Day 14 results only however, EXP2 showed a significant increase in soft tissue regeneration compared to CON, suggesting the presence of an effect of the nano controlled release system in this study on promoting oral soft tissue regeneration in critical-sized defects. The overall increase in volume in all groups at Day 3 is considered to be due to the formation of the secondary adhesion after defect formation and inflammation in the palatal region. The lack of significant difference between groups verified by the two-way ANOVA suggests that deformity was formed, in spite of the fact that hard tissues such as teeth were used as the reference stops for impression. The pressure applied at the time of impression taking for soft palatal tissue of the beagles might have caused overall deformity in some of the groups in this study. Considering various degrees of consistency in currently commercial oral impression materials, a method using a digital oral scanner can be considered in the future in order to eliminate the pressure force during the impression taking and to minimize the possibility of deformity.

In histopathological tissue specimen measurements, CON, EXP1, and EXP2 were compared at sacrifice of beagles on Day 7 and Day 14, respectively. There was no significant difference between the mean values of three groups, but a strong correlation was verified between measured values in each group up to -0.961. This suggests that there is a significant correlation between the three-dimensional measurement value of the impression scan and the two-dimensional measurement value of thickness, suggesting the efficacy of using the thickness as the representative two-dimensional soft tissue regeneration parameter in similar studies. The overall lack of significant differences between groups regarding the measured parameters could be due to the possibility of deformation in the production of tissue specimens from multiple areas on the beagles' palate.

In DNA microarray and KEGG pathway analysis, various gene expression and associated pathways between CON and EXP2 were determined in the palatal tissues of adult dogs. Ribosome is a complex of RNA and proteins that synthesize proteins in the cytoplasm. Expression of the MRPLs increased, which help protein synthesis in mitochondria. Since the RPLs are large subunits and the RPSs are small subunits, increase in the expression of both large and small subunits suggests that the expression of a majority of genes involved in the cellular processes of translation and protein synthesis increased. Ribosome biogenesis in eukaryotes begins with the synthesis of large primary RNA transcripts by RNA Polymerase I in nucleolus [16]. The expression of the following increased: FBL, which plays an important role in pre-rRNA processing [17], GAR1, a prerequisite for the telomerase complex

[18]. GTPBP4, which facilitates the migration and metastasis of cancer cells [19], NOP56, which plays an important role in the early stages of pre-rRNA processing [20], NOP58, which is related to the execution of the early pre-rRNA processing steps [21], RAN, which controls the various stages of the centrosome life cycle [22], RIOK2, which is involved in various stages of 40S maturation [23], WDR3, WDR36, WDR43, WDR43, WDR43, involved in a wide range of cellular activities, such as cell cycle progression, signal transduction, apoptosis, and gene regulation [24], and XRN2, involved in the termination of transcription [25]. Hence, the upregulated genes from the sequential release of TCA and EGF by HGC-based nano controlled release system could be considered to promote such cellular activity.

Pathway in cancer is a pathway associated with increasing cell growth, survival, invasibility and motility, and inhibiting genes that inhibit these effects [26]. The following were upregulated: CALML5, an induced protein that regulates epidermal differentiation in combination with stratifin [27], CCDC6, that reduces apoptosis [28], CXCL12, that regulates angiogenesis and several cell functions [29], DAPK2, that reduces cell damage and apoptosis [30], EDNRA, that increases proliferation and invasion of cells and decreases apoptosis [31], FGF7, involved in various cell behaviors such as mitogenicity, multiplication, motility, mobility, differentiation, and preventing cell apoptosis [32], FGFR1, that promotes cell proliferation and epithelial mesenchymal transduction (EMT) [33], FGFR2, that facilitates the proliferation, invasion, and mobility of cells [34], FZD6, that plays an important role in tumor development and transfer [35], GNG12, which regulates cell growth and casein synthesis [36], GSTA4, that plays an important role in protection against oxidative stress induced by carcinogens [37], HSP90AB1, that facilitates angiogenesis and cancer metastasis [38], JUP, that acts as intercellular attachment, strength provision and non-junction [39], KITLG, that promotes cell growth, transformation, movement, and invasion [40], LAMA4, that facilitates the survival, proliferation and movement of cells [41], LAMB1, related to the survival of neurons [42], LAMC1, involved in tissue development, tumor cell invasion and metastasis [43], MGST3, that has synthetic activations of leukotriene C4 that produces a medium for various inflammations and instantaneous hypersensitivity processes [44], MMP, that has angiogenetic activity [45], and PDGFRA, that promotes angiogenesis, cancer cell proliferation, survival, and movement [46]. However, SLC2A1, associated with the invasion and metastasis of cancer cells was downregulated [47]. The upregulated genes from the sequential release of TCA and EGF by HGC-based nano controlled release system in this study is suggested to be associated with

various activities related to the pathway in cancer. Further research is needed to determine whether SLC2A1 associated with invasion and metastasis of cancer cells showing downregulation is due to the cytotoxicity caused by the released TCA.

PI3K-Akt signaling pathway controls cell growth through protein synthesis, cell multiplication and survival [48]. The following were upregulated: COL1A1 that plays an important role in cellular adhesion and differentiation as the main components of the ECM [49], COL1A2/COL6A3, that inhibit apoptosis and promote cell proliferation, movement and invasion,⁵⁰ FGF7 [32], FGFR1 [33], FGFR2 [34], GNG12 [36], HSP90AB1 [38], ITGB5, involved in angiogenesis, metastasis, inflammation and bone absorption of cancer [51], KITLG [40], LAMA4 [41], LAMB1 [42], LAMC1 [43], PDGFRA [46], SPP1, that controls inflammation, cell adhesion, movement, differentiation, survival and bone matrix mineralization [52], and TNC, which facilitates angiogenesis, cell proliferation and movement [53]. The upregulated genes from the sequential release of TCA and EGF by HGC-based nano controlled release systems in this study is suggested to have induced the up-regulation of the PI3K-Akt signaling pathway and the genes described.

Actin cytoskeleton is closely related to cell movement, shape maintenance, polarity, tissue regeneration, angiogenesis and coupling and penetration between pathogens and host cells [54]. The following were upregulated: ARPC1A, related to cell proliferation, movement and invasion in Regulation of actin cytoskeleton [55], CFL2, which regulates the various growth factors and cytokines involved in cell growth and proliferation [56], CXCL12 [29], DIAPH3, which controls cellular mobility by reconstructing the cell skeleton [57], FGF7 [32], FGFR1 [33], FGFR2 [34], GNG12 [36], ITGB5 [51], NCKAP1, that suppresses apoptosis [58], PDGFRA [46], and PFN2, which is important for the initiation of tumors and the adjustment of metastases [59]. The upregulated genes from the sequential release of TCA and EGF by HGC-based nano controlled release in this study are considered the main cause of the up-regulation of actin cytoskeleton pathway.

Focal adhesion plays an essential role in cell growth, motility, survival, and signal transduction [60]. The following were upregulated: COL1A1 that plays an important role in cellular adhesion and differentiation as the main components of the ECM [49], COL1A2/COL6A3 [50], ITGB5 [51], LAMA4 [41], LAMB1 [42], LAMC1 [43], PDGFRA [46], SPP1 [52], and TNC [53]. Since the majority of the important genes involved in focal adhesion was up-regulated the sequential release of TCA and EGF by HGC-based nano controlled release systems in this

study could have played an essential role in triggering cell growth and survival.

The ECM acts as a barrier or filter formation, as a structural support or adhesive substrate of cells, and as an environment for sending and receiving signals. In addition, to regulate the cell function of these ECMs, the receptor provides a mechanical connection essential to the intracellular cell skeleton through the cell membrane, and communicates the signaling process between the cell space and the ECM in a complementary manner [61]. The following were upregulated: COL1A1 [49], COL1A2/COL6A3 [50], ITGB5 [51], LAMA4 [41], LAMB1 [42], LAMC1 [43], SPP1 [52], and TNC [53]. Together with the genes from the focal adhesion pathway, the upregulated genes by the sequential release of TCA and EGF played an important role in enhancing various cell behaviors.

The main function of the mitogen-activated protein kinase (MAPK) pathway is to regulate the expression of eukaryotic genes in response to extracellular signals [62]. The following were upregulated: FGF7 [32], FGFR1 [33], FGFR2 [34], GNG12 [36], HSPB1, which is promoting cell cycle [63], KITLG [40], PDGFRA [46], and STMN1, which is essential for maintaining a genomic stability during mitosis [64]. An essential cell signaling pathway related to MAPK signaling governing the cell proliferation and growth was up-regulated suggesting an importance that, despite the fact that it is cytotoxic, together with sequential release of EGF, TCA actually can promote cell proliferation and growth.

The cell cycle plays an important role in cell division, development, growth, and maintenance of life through cell division. The following were upregulated: ANAPC4 that prevent mitosis before DNA damage is recovered in G2 [65], CDK1, that initiate mitosis [66], MCM6, which is upregulated in G0 and involved in initiating DNA replication [67], PTTG1, that controls the cell cycle in the mitosis stage [68], and SFN, that affects dermal matrix component metabolism [69]. Again, the sequential release of TCA and EGF by HGC-based nano controlled release system is suggested to trigger actual cell division, proliferation and growth.

EGFR tyrosine kinase inhibitor is a treatment used in EGFR mutation-positive patients. However, within about a year of treatment, a resistance to EGFR tyrosine kinase inhibitor is developed, eventually leading to a progressive disease and resulting in cell differentiation growth [70]. The following were upregulated: FGFR2 and PDGFRA [34, 46]. The upregulation of the EGFR pathway is an obvious result from the engineered release of the EGF in this study, which also lead to the actual increase in cell proliferation and growth.

In this study, it was confirmed that the release of 0.5% TCA and EGF by HGC-based nano controlled release

system resulted in increased expression of growth factors and gene expression in *canis familiaris*. Unlike skin, the application of TCA directly to gingival area is dangerous. The potential for using HGC-based nano controlled release system guarantees safety, and at the same time, could lead to a significant increase in canine palate-based soft tissue regeneration. These results suggest that TCA, together with EGF, can be used as a tool for the development of oral soft tissue regenerative therapy. While it is necessary to clarify the clinical features in various preclinical animal models in the future, these results open a possibility of a new way to improve the regenerative effect of human gingival tissues.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The animal experiment protocol was approved by the Institutional Animal Care and Use Committee, Cronex, Hwasung-si, Gyeonggi-do, Republic of Korea (CRONEX-IACUC: 201803002).

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