The Effect of T3, EGF and HB-EGF on Human Periodontal Fibroblasts

Eun Kyoung Hong

The Graduate School
Yonsei University
Department of Dental Science

The Effect of T3, EGF and HB-EGF on Human Periodontal Fibroblasts.

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Eun Kyoung Hong

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Eun Kyoung Hong

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Abstract

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Eun Kyoung Hong

Department of Dental Science, The Graduate School,

Yonsei University

(Directed by professor Seong-Oh Kim)

Viable cells of periodontal ligament would be an important factor for the successful replantation of an avulsed tooth. Therefore, it is critical to choose the storage medium for the preservation of traumatically avulsed teeth.

Growth factors and hormones could be considered for the therapeutic application of the maintenance of viable periodontal ligament fibroblasts(PDLFs). The positive effects of tri-iodothyronine(T3), epidermal growth factor(EGF) and heparine-binding epidermal-like growth factor(HB-EGF) on other tissues have been studied previously. Therefore, T3, EGF and HB-EGF were examined to evaluate the therapeutic application on avulsed teeth.

The cell proliferation of PDLFs was increased dose-dependently in the

presence of T3, EGF and HB-EGF. Maximum cellular growth was shown at the concentration of 10 nM T3, 10 ng/ml EGF and 10 nM HB-EGF. Interestingly, HB-EGF showed maximum effect on the proliferation of PDLFs, and it was suggesting that HB-EGF might be a choice of candidate for a therapeutic application.

Combined effects of these factors on the proliferation of PDLFs were also studied. Combination of T3, EGF and HB-EGF enhanced cellular growth than that of a single growth factor showing synergistic effect.

Especially, combination of T3 and HB-EGF enhanced the proliferation of PDLFs with statistical significance. I studied the effect of T3 on endogenous EGF receptor using RT-PCR. The result was that T3 enhanced the expression of EGF receptors of PDLFs.

From this study, it is suggested that HB-EGF or the combination of T3 and HB-EGF could be used as a pretreatment agent for traumatically avulsed teeth.

Key words: T3, EGF, HB-EGF, PDLFs, tooth avulsion

The Effect of T3, EGF and HB-EGF on Human Periodontal Fibroblasts

<Directed by professor Seong Oh Kim, DDS, PhD>

Eun Kyoung Hong

Department of Dental Science, The Graduate School,

Yonsei University

I. Introduction

Tooth avulsion constitutes 0.5-16% of all traumatic injuries in permanent anterior teeth. Successful replantation of an avulsed tooth may depend on the presence of viable cells in periodontal ligament, which can proliferate on denuded root surface. Therefore, in case of the periodontal injury by tooth avulsion, maintenance of viable periodontal ligament fibroblasts (PDLFs) would be one of the most important factors for the good prognosis.

During extra alveolar period, storage medium could be used not only to preserve the PDLFs, but also to stimulate their growth by adding growth factors. Polypeptide growth factors are a class of natural biological mediators that regulate the proliferation, differentiation, migration, and

matrix synthesis of cells. The growth factors studied nowadays are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) and insulin-like growth factors (IGF I & II). Recently, it has been reported that polypeptide growth factors enhanced the formation of bone, dentin, and collagen fibers in periodontal wounds (Giannobile, 1996; Narayanan and Bartold, 1996). Lynch *et al.* demonstrated that short-term application of a combination of PDGF-B and IGF could enhance the formation of periodontal attachment apparatus by 5-10 folds during the early phase of wound healing after surgery. Two growth factors synergistically accelerated the healing of partial thickness skin wounds and the regeneration of periodontal tissues. Therefore, growth factors could be used as a locally acting therapeutic agent to preserve PDLFs after avulsive dental injury.

Hormones systemically regulate growth and metabolism of body whereas growth factors locally take effect on tissue. In the developing animal and human, thyroid hormones play a critical role in development and differentiation of tissues and organs (Jannini et al., 1995; Metz et al., 1996; Krassas, 2000). Kim (2001) reported a patient showing remarkably rapid tooth movement of forced eruption procedure during temporary hyperthyroid period. He said that there was a close relationship between dental eruption rate and serum level of thyroid hormone. Park (2003) reported that

exogenous tri-iodothyronine (T3) injection raised the turnover rate of cells in the periodontal ligament is well known that T3 interact with other hormones such as growth hormones and steroid hormones.

EGF is the first tissue-derived peptide enhancing proliferation and differentiation of cells. EGF was extracted from submaxillary glands when injected into newborn animals, it induced precocious tooth eruption and premature eyelid opening (Cohens, 1962). In human, most of circulating EGF is associated with blood platelets, synthesized by megakaryocytes, and released in the process of blood coagulation. Locally EGF is also secreted from parotid and submandibular glands, and released directly into saliva (Mattilla, 1987).

Heparine-binding epidermal-like growth factor (HB-EGF) was first identified as a 20-22 KD glycoprotein in conditioned medium of macrophage-like cells (Cook, 1995). Similar to other EGF-family growth factors, HB-EGF binds to the epidermal growth factor receptor (EGFR), thereby inducing its phosphorylation and stimulates DNA synthesis in target cells (Raab, 1997). HB-EGF plays an important role in myogenesis, mucosal repair of stomach, protecting the small bowl from ischemic injury, pancreatic development, vascular remodeling, renal cell repair and proliferation, liver regeneration and wound healing (Karen, 1998).

The purpose of this study was to evaluate the effect and the interaction of T3, EGF and HB-EGF on the proliferation of PDLFs and to suggest the possibility of therapeutical application of for the preservation of PDLFs in a traumatically avulsed tooth.

II. Materials and Methods

1. Experimental materials

1) T3

T3 was obtained from Sigma-Aldrich Co. and dissolved in 1 N NaOH to a concentration of 1 mg/ml. It was diluted with 10% phosphate buffered saline (PBS) making 10^{-5} M stock solution and stored at –20 ℃. It was further diluted to several appropriate concentrations just before experiment.

2) EGF

Easyf® (Daewoong Pharm Co. Ltd. Korea) is the biochemical synthetic material which has the same structure and equivalent activity to human EGF. For *in vitro* study, it was dissolved with 10% phosphate buffered saline (PBS) to a concentration of 10^{-6} g/ml for the stock solution. It was then diluted in PBS to several appropriate concentrations just before the experiment.

3) HB-EGF

HB-EGF was obtained from R & D Co. and dissolved with 10% PBS to a

concentration of 5 μg/ml for the stock solution. It was then diluted to several appropriate concentrations for the experiment.

4) Periodontal ligament fibroblasts (PDLFs)

Human PDLFs were obtained and cultured from the explant tissue of human healthy periodontal ligament taken from several first premolars that were extracted for orthodontic reasons.

2. Experimental methods

1) Cell culture

After removing calculus and plaque in mouth, the premolar was extracted and rinsed 3 times with Hanks' balanced salt solution (HBSS) to remove the blood clot of root surface. The periodontal ligament tissues from the middle third of the roots were minced, put in culture dishes and incubated in α-MEM with 10% fetal bovine serum (FBS) and antibiotics of 100 mg/ml streptomycin, 0.5 mg/ml amphotericin-B, and 100 unit/ml penicillin at 37 ℃ in a humidified atmosphere of 5% CO_2-95% air. The media was changed every 3 days until dense single layer was gained. After cells reached confluence, they were trypsinized with 0.25% trypsin-EDTA in PBS for

subculture. All the experiments were done within 5-10 cellular passages.

2) Bioassay of T3, EGF and HB-EGF

3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, was used to evaluate the viable cell proliferation. MTT assay is based on the principle of tetrazolium salt being reduced by mitochondrial reducing enzyme (succinate dehydrogenase) so that the toxicity of viable cells and cellular differentiations could be evaluated. The reduced tetrazolium salt is converted into colored water-insoluble formazan salt, and it can be evaluated spectrophotometrically once the MTT-formazan is dissolved in an organic solvent.

About 2000 PDLFs were seeded and grown on the 96 well plate in 200 $\mu\ell$ α-MEM/2% FBS for 1day. Then the culture media was replaced with 200 $\mu\ell$ α-MEM containing T3, EGF and HB-EGF at several concentrations: T3 at the concentrations of 0, 0.1, 1, 10, 50 and 100 nM, EGF at the concentrations of 0, 0.1, 0.5, 1, 10 and 100 ng/ml, HB-EGF at the concentrations of 0, 0.01, 0.1, 1, 10 and 50 nM. Cell proliferation was measured by MTT assay after 3 days and 5 days of culture period. 50 $\mu\ell$ of MTT solution (5mg/ml) was added per well then incubated at 37 ℃ in incubator for 4 hrs. The purple formazan product was dissolved in 150 $\mu\ell$

of dimethylsulfoxide (DMSO) and incubated for 30 minutes at 37 ℃ in CO_2 incubator. Optical density was measured on an ELISA reader at 570 nm.

3) Bioassay of mixture (T3, EGF and HB-EGF)

The MTT assay was proceeded as described above 2). Combination of factors with several concentrations is listed in Table 1.

Table 1. The groups of combination of T3, EGF and HB-EGF

Group	Combinations
Group 1	0.1 nM T3 + 0.01, 0.1 and 1 ng/ml EGF
Group 2	1 nM T3 + 0.01, 0.1 and 1 ng/ml EGF
Group 3	10 nM T3 + 0.01, 0.1 and 1 ng/ml EGF
Group 4	0.1 nM T3 + 0.01, 0.1 and 1 nM HB-EGF
Group 5	1 nM T3 + 0.01, 0.1 and 1 nM HB-EGF
Group 6	10 nM T3 + 0.01, 0.1 and 1 nM HB-EGF
Group 7	0.01 nM HB-EGF + 0.01, 0.1 and 1 ng/ml EGF
Group 8	0.01 nM HB-EGF + 0.01, 0.1 and 1 ng/ml EGF

4) RT-PCR

About $6x10^5$ PDLFs were plated on 100x15mm (100π) dishes in α-

MEM/2% FBS and were incubated overnight to allow attachment prior to the addition of T3, EGF, and HB-EGF. After 24hrs of incubation period, α-MEM solutions containing (a) 0.1 nM T3, (b) 10 nM T3, (c) 0.1 nM T3 + 0.1 nM HB-EGF, (d) 0.1 nM T3 + 0.1 ng/ml EGF, were replaced in experimental group and were replaced in control group with same media without T3, EGF, and HB-EGF. After 1 day of incubation, cells of 5 groups were harvested by Trizol. And then DNA was removed with chloroform and salted out with isopropanol and 70% ethanol. After centrifuging, mRNA pellet was dried then dissolved in diethyl pyrocarbonate (DEPC) water. They were stored at -70℃. After mRNA was quantified by spectrophotometry, cDNA was synthesized by reverse trascriptase with oligo dT primer. cDNA was used as a template for PCR to amplify the specific product. The sequences of each set of primers are listed below:

EGFR P1, 5'-ATGCGACCCTCCGGGACGGCCG-3', EGFR P2, 5'-CCTTCAGTCCGGTTTTATTTGC-3'. PCR was proceeded with AccuPower® RT PreMIX tube. RT-PCR products were separated by 1% agarose gel electrophoresis in 0.5% tris-acetate (TAE) buffer and stained with ethidium bromide. All data were quantitated relative to β-actin genes. Data analysis was performed with Spot Denso Analysis of Frog 2000 software.

5) Statistical analysis

Statistical analysis was done with statistical software (SAS version 8.1). Absorbance of each concentration was tested by the analysis of Tukey's multiple comparison tests at 95% confidence level.

III. Result

1. Bioassay of T3, EGF, and HB-EG

The purpose of this study was to evaluate the effect of T3, EGF and HB-EGF on the proliferation of PDLFs. In the group of T3, there was different cellular proliferation between control and 10 nM T3; 0.1 nM T3 and 10 nM T3 on the 5th day with statistical significance ($p<0.05$). In addition, the proliferation of PDLFs in 10 nM T3 on the 3rd day was increased up to 31.2% when compared with control. The proliferation of PDLFs in the 10 nM T3 on the 5th day was increased up to 54.0% when compared with control (Table 2, Fig. 1)

Table 2. The effect of T3 on the proliferation of hPDLFs. The proliferation of PDLFs was measured by MTT assay after the treatment of T3 for 3 and 5 days.

Concentration of T3 (nM)	Absorbance *(Mean ± SD)*	
	after 3 days	after 5 days
0	*0.301 ± 0.051*	*0.339± 0.037**
0.1	*0.327 ± 0.06*	*0.342 ± 0.020*†
1	*0.338 ± 0.120*	*0.414 ± 0.061*
10	*0.395 ± 0.054*	*0.522 ± 0.090**†
50	*0.356 ± 0.077*	*0.441 ± 0.030*
100	*0.321 ± 0.067*	*0.421 ± 0.056*

*,† Statistically significant (p<0.05)

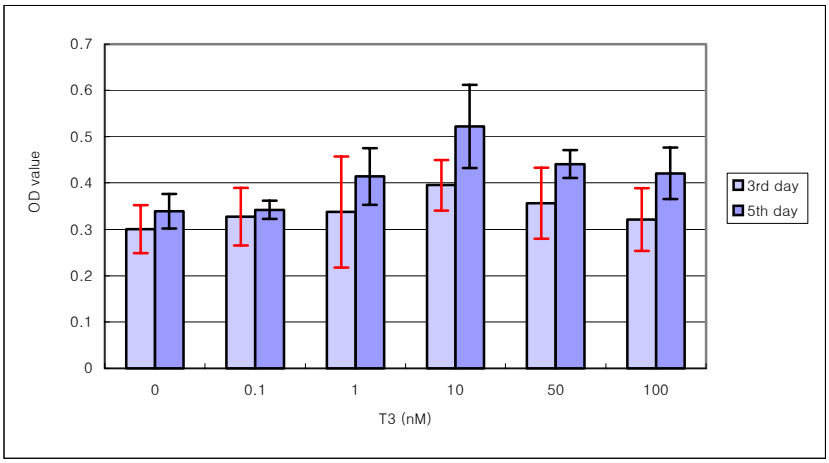

Fig. 1. The proliferation of PDLFs with several T3 concentrations

In the treatment of EGF, there was different cellular proliferation between control and 10 ng/ml ; control and 100 ng/ml on the 3rd day with statistical significance ($p<0.05$). In addition, the proliferation of PDLFs in 10 nM EGF on the 3rd day was increased up to 32.3% when compared with control, and the proliferation of PDLFs in 10 nM EGF on the 5th day was increased up to 41.3% when compared with control (Table 3, Fig. 2).

Table 3. The effect of EGF on the proliferation of hPDLFs. The proliferation of PDLFs was measured by MTT assay after the treatment of EGF for 3 and 5 days.

Concentration of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
	after 3 days	after 5 days
0	*0.368± 0.072*	*0.356 ± 0.054*
0.1	*0.380 ± 0.022*	*0.385 ± 0.038*
0.5	*0.392 ± 0.053*	*0.405 ± 0.035*
1	*0.442 ± 0.051*	*0.434 ± 0.047*
10	*0.487 ± 0.023**	*0.503 ± 0.044*
100	*0.348 ± 0.081**	*0.413 ± 0.034*

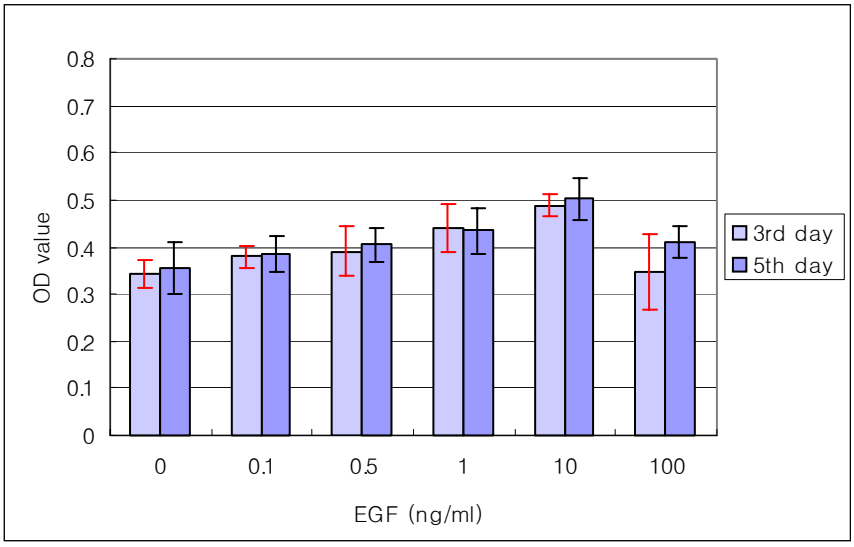

Fig. 2. The proliferation of PDLFs with several EGF concentrations

In the treatment of HB-EGF, the highest degree of proliferation of PDLFs was at the concentration of 10 nM HB-EGF on the 5th day (Table 4, Fig. 3). The proliferation of PDLFs in the 10 nM HB-EGF on the 3rd day was increased up to 33.5% when compared with control. The proliferation of PDLFs in the 10 nM HB-EGF on the 5th day was increased up to 83.6% when compared with control.

Table 4. The effect of HB-EGF on the proliferation of hPDLFs. The proliferation of PDLFs was measured by MTT assay after the treatment of HB-EGF for 3 and 5 days.

Concentration of HB-EGF (nM)	Absorbance *(Mean ± SD)*	
	after 3 days	after 5 days
0	*0.343± 0.022**	*0.366± 0.059**
0.01	*0.317 ± 0.033*	*0.360 ± 0.041*†
0.1	*0.386 ± 0.042*	*0.400± 0.050*‡
1	*0.408± 0.017*	*0.454± 0.093*∫
10	*0.458± 0.047**†	*0.672± 0.063**†‡∫∬
50	*0.332 ± 0.042*†	*0.459± 0.060*∬

*,†,‡,∫, ∬ Statistically significant (p<0.05)

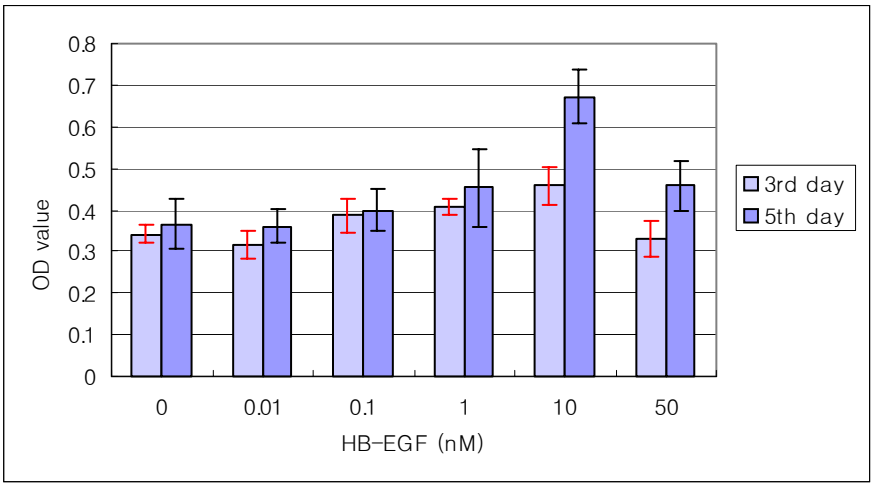

Fig. 3. The proliferation of PDLFs with several HB-EGF concentrations

From MTT assay, I concluded that maximum cellular proliferation was reached at the concentrations of 10 nM T3, 10 ng/ml EGF and 10 nM HB-EGF on the 3rd and the 5th day.

2. Bioassay in the combination of T3, EGF and HB-EGF

In this study, the interaction of T3, EGF and HB-EGF were evaluated. The combination of 0.1 nM T3 and EGF at the two different concentrations (0.1 ng/ml and 1 ng/ml) on the 5th day, showed higher value of MTT assay than those of T3 alone or EGF alone (Table 5, Fig. 4). But there was no statistical significance. The proliferation of PDLFs was increased more in the group of combination compared with the single treatment. The proliferation of PDLFs was increased dose-dependently in the group of combined treatment.

Table 5. The effect of combined treatment with 0.1 nM T3 and several different concentrations of EGF on the proliferation of hPDLFs.

Concentration of T3 (nM)	Concentration of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
0.1	0.01	*0.343± 0.073*	*0.433± 0.063*
0.1	0.1	*0.349± 0.007*	*0.455± 0.050*
0.1	1	*0.354± 0.017*	*0.505± 0.089*

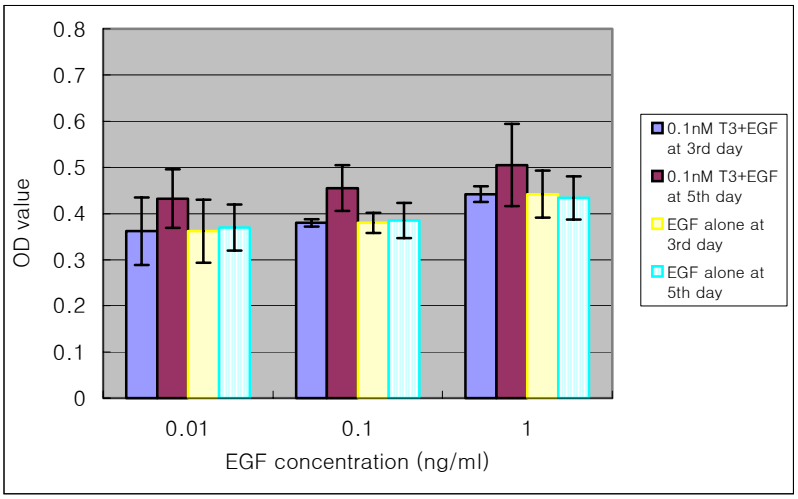

Fig. 4. The proliferation of PDLFs with the combination of 0.1 nM T3 and several different concentrations of EGF

The proliferation of PDLFs was increased in the group of combination compared with the group of single treatment (Table 6, Fig. 5). The combination of 1 nM T3 and EGF at every concentrations on the 3rd and the 5th day, showed higher value of MTT assay than those of the treatment of T3 alone or EGF alone.

Table 6. The effect of combined treatment with 1 nM T3 and several different concentrations of EGF on the proliferation of hPDLFs.

Concentration of T3 (nM)	Concentration of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
1	0.01	*0.377± 0.089*	*0.413± 0.024*
1	0.1	*0.413± 0.035*	*0.413± 0.050*
1	1	*0.411± 0.023*	*0.432± 0.060*

There was no statistical significance ($p<0.05$).

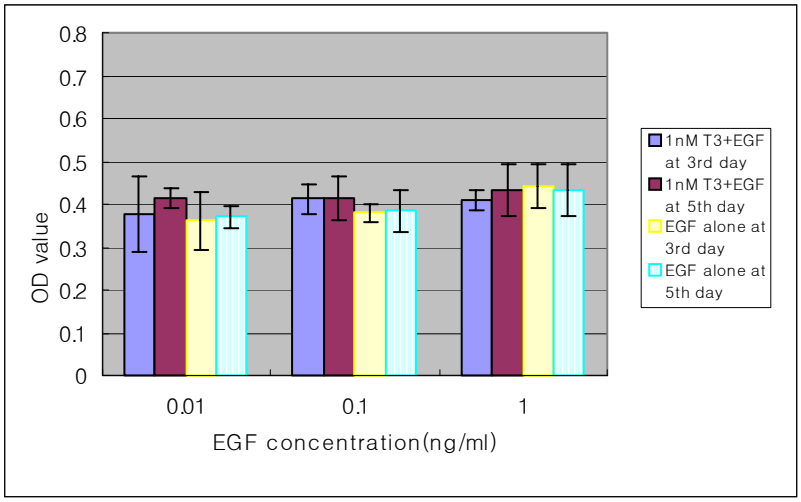

Fig. 5. The proliferation of PDLFs on the concentration of 1 nM T3 and several different concentrations of EGF

The proliferation of PDLFs was increased in the group of combination compared with that in the single treatment. The combination of 10 nM T3, and EGF at every concentrations on the 3rd and the 5th day, showed higher value of MTT assay than those of the treatment of T3 alone or EGF alone (Table 7, Fig 6). The proliferation of PDLFs was increased dose-dependently in the group of combined treatment, except for the 1 ng/ml EGF on the 5th day.

Table 7. The effect of combined treatment with 10 nM T3 and several different concentrations of EGF on the proliferation of hPDLFs.

Concentration of T3 (nM)	Concentration Of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	After 5 days
10	0.01	*0.410± 0.032*	*0.480± 0.111*
10	0.1	*0.411± 0.041*	*0.571± 0.161*
10	1	*0.419± 0.045*	*0.490± 0.071*

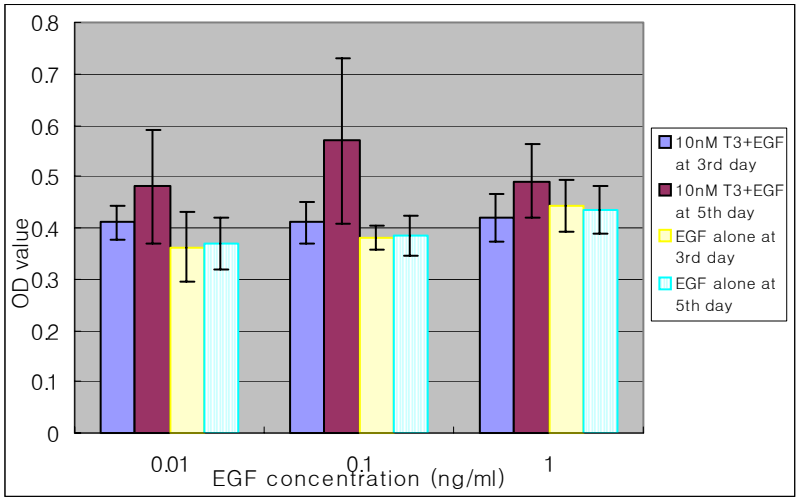

Fig. 6. The proliferation of PDLFs with the combination of 10nM T3 and several different concentrations of EGF

The combination of T3 and HB-EGF significantly enhanced the proliferation of PDLFs (p<0.05). The combined treatment of T3 and HB-EGF resulted in better cellular growth than that of HB-EGF alone or T3 alone. The combination of 0.1 nM T 3 and HB-EGF at every concentrations on the 3rd and the 5th day (except for 1 nM HB-EGF on the 3rd day), showed higher value of MTT assay than that of the treatment of T3 alone or HB-EGF alone (Table 8, Fig. 7). The cellular proliferation was increased dose-dependently with the combined treatment, except for 1 nM HB-EGF on the 3rd day.

Table 8. The effect of combined treatment with 0.1 nM T3 and several different concentrations of HB-EGF on the proliferation of hPDLFs.

Concentration of T3 (nM)	Concentration of HB-EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
0.1	0.01	*0.356± 0.041*	*0.445± 0.036**
0.1	0.1	*0.370± 0.012*	*0.456± 0.011*
0.1	1	*0.365± 0.026*	*0.500± 0.049**

*Statistically significant (p<0.05)

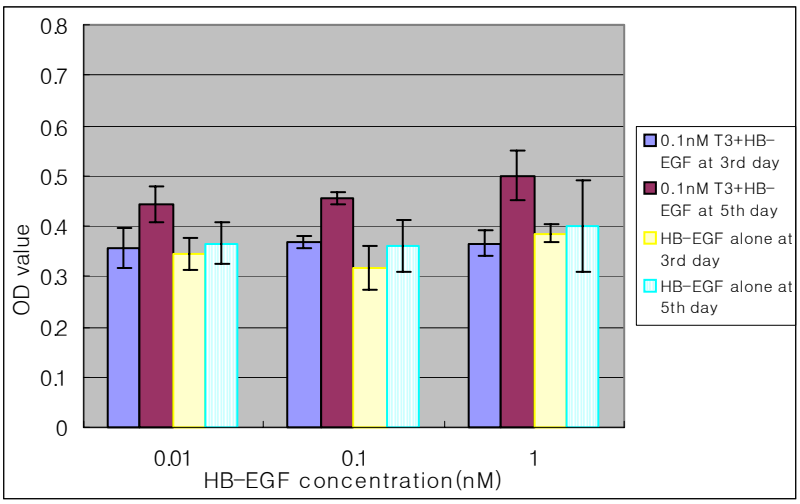

Fig. 7. The proliferation of PDLFs with the combination of 0.1 nM T3 and several different concentration of HB-EGF

The proliferation of PDLFs was increased in the group of combination compared with the single treatment. The combination of 1 nM T3 and HB-EGF at every concentrations on the 3rd and the 5th day, showed higher value of MTT assay than those of the treatment of T3 alone or HB-EGF alone (Table 9, Fig. 8). The proliferation of PDLFs was increased dose-dependently in the combined treatment, except for 1 nM HB-EGF on the 3rd day.

Table 9. The effect of combined treatment with 1 nM T3 and several different concentrations of HB-EGF on the proliferation of hPDLFs (p<0.05)

Concentration of T3 (nM)	Concentration of HB-EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
1	0.01	*0.385± 0.016*	*0.432± 0.026**
1	0.1	*0.417± 0.040*	*0.441± 0.031*†
1	1	*0.397± 0.066*	*0.516± 0.027**†

*,† Statistically significant (p<0.05)

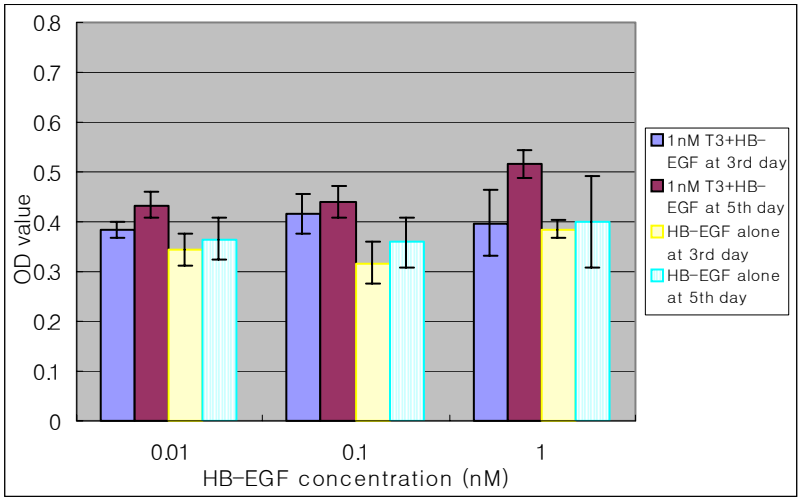

Fig. 8. The proliferation of PDLFs with the combination of 1 nM T3 and several different concentrations of HB-EGF

The proliferation of PDLFs was increased in the group of combination compared with the single treatment. The combination of 10 nM T3 and HB-EGF at every the concentrations on the 3rd and the 5th day, showed higher value of MTT assay than those of the treatment of T3 alone or HB-EGF alone. The proliferation of PDLFs was increased dose-dependently in the combined treatment. At the 5th day, the maximum proliferation of PDLFs was observed with the combination of 1 nM HB-EGF and 10 nM T3 (Table 10, Fig. 9).

Table 10. The effect of combined treatment with T3 10 nM and several different concentrations of HB-EGF on the proliferation of hPDLFs ($p<0.05$)

Concentration Of T3 (nM)	Concentration of HB-EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
10	0.01	*0.375± 0.024*	*0.471± 0.039**
10	0.1	*0.392± 0.020*	*0.509± 0.037*†
10	1	*0.444± 0.059*	*0.661± 0.052**†

*,† Statistically significant ($p<0.05$)

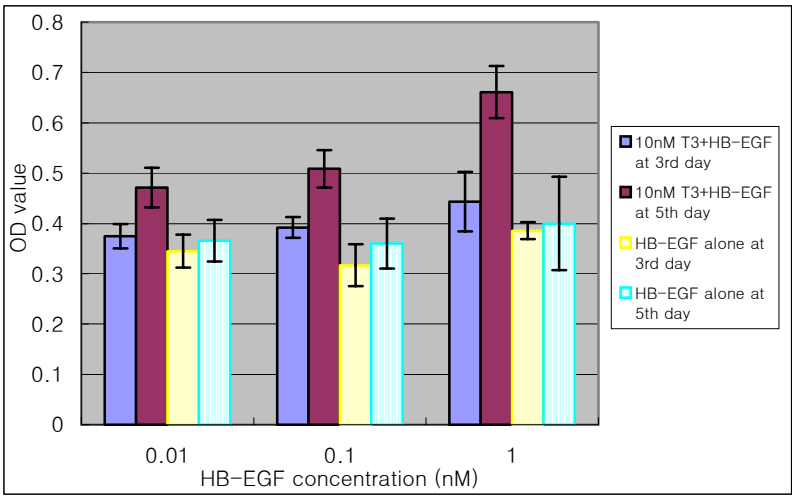

Fig. 9. The proliferation of PDLFs with the combination of 10 nM T3 and several different concentrations of HB-EGF

The proliferation of PDLFs was increased in the group of combination compared with the single treatment. The combination of 0.01 nM HB-EGF and EGF at every concentrations on the 5th day, showed higher value of MTT assay than those of the treatment of HB-EGF or EGF alone (Table 11, Fig. 10). The proliferation of PDLFs was increased dose-dependently in the combined treatment, except for 0.1 nM HB-EGF on the 5th day.

Table 11. The effect of combined treatment with HB-EGF 0.01 nM and several different concentrations of EGF on the proliferation of hPDLFs.

Concentration of HB-EGF (nM)	Concentration of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	after 5 days
0.01	0.01	*0.344± 0.013*	*0.441± 0.056*
0.01	0.1	*0.350± 0.008*	*0.440± 0.052*
0.01	1	*0.361± 0.019*	*0.472± 0.076*

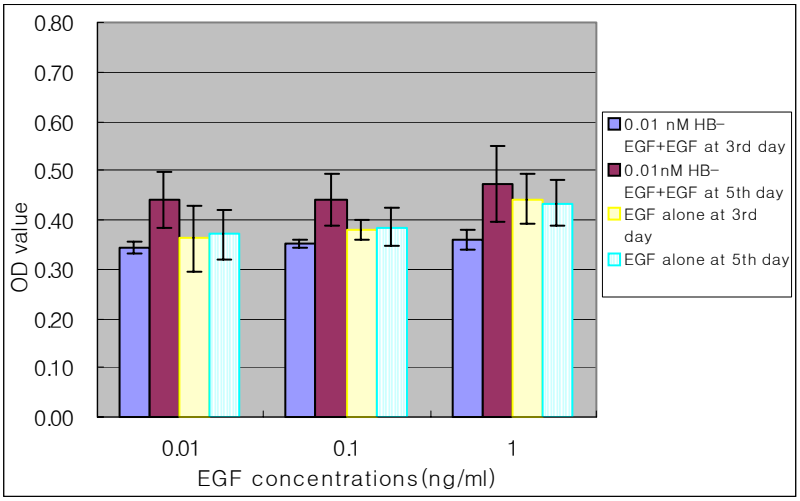

Fig. 10. The proliferation of PDLFs with the combination of 0.01 nM HB-EGF and several different concentrations of EGF

The proliferation of PDLFs was increased in the group of combination compared with the single treatment. The combination of 0.1 nM HB-EGF and EGF at every concentrations on the 5th day, showed higher value of MTT assay than those of the treatment of HB-EGF or EGF alone (Table 12, Fig. 11). The proliferation of PDLFs was increased dose-dependently in the combined treatment on the 5th day.

Table 12. The effect of combined treatment with 0.1 nM HB-EGF and several different concentrations of EGF on the proliferation of hPDLFs.

Concentration of HB-EGF (nM)	Concentration of EGF (ng/ml)	Absorbance *(Mean ± SD)*	
		after 3 days	After 5 days
0.1	0.01	*0.335± 0.033*	*0.424± 0.039**
0.1	0.1	*0.336± 0.028*	*0.460± 0.033*
0.1	1	*0.349± 0.008*	*0.513± 0.025**

* Statistically significant (p<0.05)

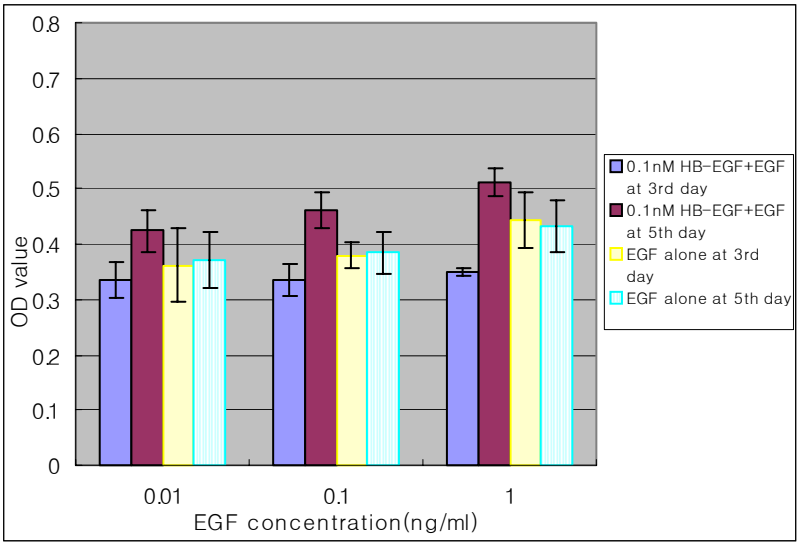

Fig. 11. The proliferation of PDLFs with the combination of 0.1 nM HB-EGF and several different concentrations of EGF

3. RT-PCR

Using RT-PCR, I tested if the addition of T3, EGF and HB-EGF had an effect on mRNA expression of endogenous EGFR in PDLFs. All results were expressed as relative units and standardized to the amount of β-actin mRNA. In the treatment of 0.1 nM T3, EGFR expression was increased up to 5.6 folds when compared with control (Table 13). T3 and the combination of T3 and EGF or HB-EGF enhanced the expression amount of EGFR.

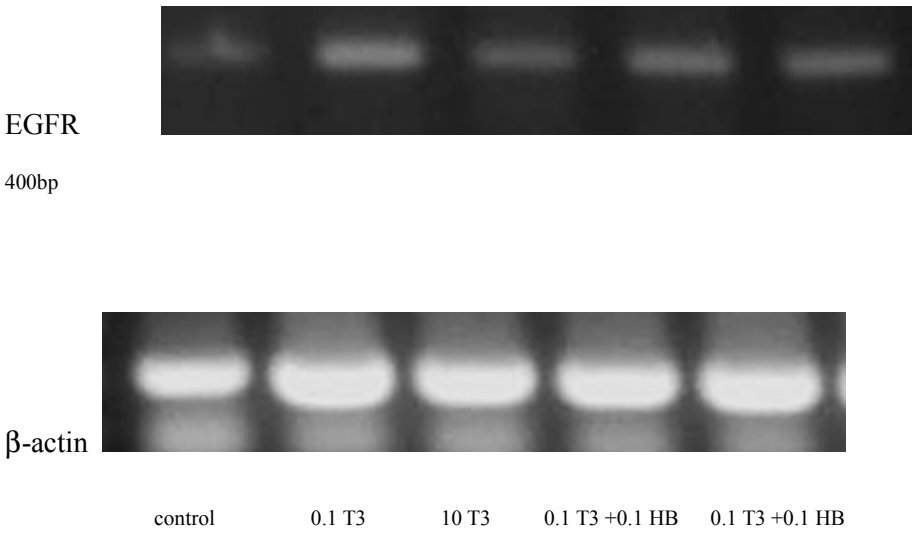

Fig. 12. The expression amount of EGFR using RT-PCR

Table 13. The ratio of EGFR between control and experimental group

	EGFR / β-actin (ratio of area)	Experimental group / control group
control	0.157	1
a) 0.1 nM T3	0.877	5.6
b) 10 nM T3	0.394	2.5
c) 0.1 nM T3 + 0.1 nM HB-EGF	0.655	4.2
d) 0.1 nM T3 + 0.1 nM EGF	0.528	3.4

IV. Discussion

Periodontal ligament is a dense connective tissue between root cementum and alveolar bone that anchors the tooth and maintains the structural integrity of mineralized tissues. Fibroblasts in the periodontal ligament have multipotential heterogenous ability that can differentiate into either cementoblasts or osteoblasts.

An avulsion may cause the damage of attachment apparatus in periodontal ligament. Thus it is important to maintain the viable cell of periodontal ligament attached to the avulsed tooth for the successful replantation.

Ideally, the tooth should be taken good care immediately after the injury in an effort to preserve the viability of PDLFs, and thereby to optimize healing process and minimize root resorption. Therefore, the storage conditions should be designed to maximize preservation of PDLFs during the transportation to dental office when immediate replantation of an avulsed tooth is impossible.

The optimal storage medium should be able to preserve the viability, mitogenicity and clogenic capacity of injured PDLFs and their progenitors. Numerous studies have shown that storage conditions and types of storage media affect the viability of PDLFs. Recently, three media have been

introduced to preserve avulsed teeth : Hanks' Balanced Salt Solution (HBSS), Viaspan, and fibroblast culture media (α-MEM). Because of the limitation of available conventional storage media to obtain successful regeneration of injured PDL tissues, the application of growth factors have been attempted in clinical use to accelerate the proliferation of regeneration of PDLFs, which may locally help tissue repair.

The recent studies have been reported that the addition of polypeptide growth factor into storage media showed better effect on the regeneration of PDLFs. For example, *in vivo* and *in vitro* studies have revealed that PDGF-B and IGF-1 have the potential to enhance the periodontal regeneration. In particular, the combined use of these growth factors induces synergistically the regeneration of the periodontal ligament as well as bone and cementum (Lynch et al, 1991).

Numerous growth factors have been reported to influence, either positively or negatively. EGF has been proven to be important in the process of wound healing. PDL cells, including fibroblasts, preosteoblasts, and epitherial rests of Malassez cells, express numerous EGF receptors responding to EGF.

Thyroid hormone modulates the transduction of signals from several cytokines and growth factors in human cell lines. Also, thyroid hormone enhances the effects of EGF on mitogen activated protein kinase (MAPK)

activation and protooncogene expression (Lin, 1999). And the effect of EGF on immediate-early gene expression was enhanced by thyroid hormone. Physiologic levels of thyroid hormone may enhance the autocrine/paracrine effects of EGF on cells. In other study, it has been reported that genomic cellular actions of thyroid hormone include increased expression of the EGF gene (Rogers SA, 1995). Likely euthyroid organisms, therefore, many cells secret these growth factors in response to physiologic levels of thyroid hormone (Lin H-Y, 1999).

In this study, the effects of T3, EGF and HB-EGF on the proliferation of PDLFs and the synergic interaction of these factors were examined to evaluate the potential application of T3, EGF and HB-EGF as a storage media or a pre-replantation conditioner of avulsed tooth. Cell proliferation of PDLF was increased dose-dependently in the presence of T3, EGF and HB-EGF.

The optimal concentration of T3, EGF and HB-EGF in the cellular growth was reached at 10 nM T3, 10 ng/ml EGF and 10 nM HB-EGF. Interestingly, the effect of HB-EGF on the proliferation of PDLFs was the highest, suggesting that HB-EGF could be a good candidate for a therapeutic application.

In this study, the result from T3 at the optimal concentration of 10 nM was

close to that from the combination of low concentration of T3 and EGF or HB-EGF. In group 1, the combination of T3 at the concentration of 0.1 nM and EGF at the concentration of 0.1 ng/ml on the 5^{th} day, showed increased value of MTT assay compared with the group of EGF alone (except the group of 10 nM EGF) in spite of the low concentration (Table 5, Fig. 7). In particular, the combination of T3 and HB-EGF was effective on cellular proliferation. In group 5 and group 6, I found effective cellular proliferation with significance (Table 9, 10 , Fig. 10, 11).

T3 modulates EGF-induced renal proximal tubule cell proliferation by an effect on the EGF receptor (Humes HD, 1992). He concluded that T3 enhanced EGF receptor gene expression increasing the number of cell surface EGF receptors on renal proximal tubule cells. A potentiated mitogenic response to EGF may be a mechanism for thyroid hormone to enhance renal functional recovery following toxic acute renal failure.

In this study, using RT-PCR, I tested if the addition of T3, EGF and HB-EGF had an effect on mRNA expression of endogenous EGFR in PDLFs. As a result, T3 enhanced the expression of EGFR in PDLFs. All results were expressed as relative units and standardized to the amount of β-actin mRNA. In 0.1nM T3 treated group, EGFR expression was increased up to 5.6 folds compared with control. Also, in the combination group of T3 at the

concentration of 0.1 nM and HB-EGF at the concentration of 0.1 nM, the EGFR expression was increased 4.2 folds compared with control. I could conclude that T3 induces EGF receptor gene expression in PDLFs. Therefore, in the combination of T3, EGF and HB-EGF even at low concentration, cell proliferation was increased by binding of EGF and HB-EGF to the increased EGFR. Also, the combined application of T3 and HB-EGF could enhance the proliferation of PDLFs because interaction between HB-EGF and EGFR was increased by T3.

From this study I could conclude that T3, EGF and HB-EGF enhanced the proliferation of PDLFs, which could be used as a pretreatment agent for traumatically avulsed teeth. Further study is needed to find out the relationship between these specific growth factors and matrix synthesis by PDLFs. Also, we need to proceed *in vivo* experiment using HB-EGF and the combination of T3 and HB-EGF.

V. Conclusion

In this study, I evaluated the effect and interaction of T3, EGF and HB-EGF on the proliferation of PDLFs by MTT assay. To understand the synergic effect of T3 and HB-EGF, RT-PCR on EGF receptor was done considering the influence of T3.

The results are summarized as;

1. The optimal concentration of T3, EGF and HB-EGF in cellular growth by MTT assay was found at the concentrations of 10 nM T3, 10 ng/ml EGF and 10 nM HB-EGF.

2. Maximum proliferation of PDLFs was found in HB-EGF group at the concentration of 10 nM with statistical significance ($p<0.05$).

3. The combination of T3 and HB-EGF enhanced the proliferation of PDLFs with statistical significance ($p<0.05$).

4. The expression of EGFR increased with added T3.

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국문요약

갑상선호르몬과 표피성장인자, 헤파린결합 표피성장인자가 치주인대세포에 미치는 영향

완전탈구 된 치아의 예후는 살아있는 치주인대세포의 영향을 받으며, 외상으로 완전탈구 된 치아를 저장하는 보관액은 재식 성공의 예후에 중요한 요소이다. 따라서, 최근에 치주인대세포의 생존을 증가시키기 위한 전처리제로서 성장인자와 호르몬에 대한 연구가 진행되고 있다. 이들 중 갑상선호르몬 (이하 T3)과 표피성장인자 (이하 EGF)및 헤파린결합 표피성장인자 (이하 HB-EGF)가 여러 기관의 세포 증식을 활성화 시킨다는 많은 보고가 있다. 이 연구의 목적은 갑상선호르몬, 표피성장인자, 헤파린결합 표피성장인자가 세포의 증식에 미치는 영향을 살펴보는 것이며, 이 상호 작용이 섬유모세포의 증식에 미치는 영향을 알아보는 것이다. 또한, 성장인자들의 조합이 표피성장인자 수용체의 발현에 미치는 영향을 알아보고자 한다.

실험결과 T3와 EGF및 HB-EGF로 처리한 경우, 최대 증식을 보이는 농도까지는 농도에 비례하여 증가하였다. 섬유모세포의 최대 성장을 보이는 T3의 농도는 10 nM이었으며, EGF는 10ng/ml, HB-EGF는 10 nM 이었다. 주목할 것은 특히 10 nM의 HB-EGF를

처리한 경우, 치주인대 섬유모세포의 증식이 가장 컸다.

T3와 EGF및 HB-EGF을 조합하여 처리한 경우 한가지 성장인자만을 처리했을 때보다 세포 증식에 있어서 보다 효과적이었다. 특히 T3와 HB-EGF의 조합은 섬유모세포 증식을 유의하게 향상시켰다. RT-PCR (reverse transcriptase polymer chain reaction)로 표피성장인자 수용체(EGFR)의 발현율을 관찰한 결과, T3가 EGFR의 발현을 증가시킴을 알 수 있었다. 따라서 T3의 표피성장인자에 대한 발현 증식 작용과 이에 결합하는 HB-EGF의 상승작용으로 이들을 조합한 경우, 세포 증식이 증가되었음을 유추할 수 있었다.

이번 연구를 통해 HB-EGF를 단독 사용한 경우와 T3와 HB-EGF를 조합한 경우가 효과적으로 섬유모세포의 증식을 향상시키고, 완전탈구 된 치아의 전 처리제로서 유용하다는 결론을 내릴 수 있었다.

핵심되는말 : 갑상선호르몬, 표피성장인자, 헤파린결합 표피성장인자, 치주인대섬유모세포, 완전탈구