The Effect of Thyroid Hormone on the Cell Proliferation of Periodontal Ligament in Rat

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Dong Suk Park

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Abstract

The Effect of Thyroid Hormone on the Cell Proliferation of Periodontal Ligament in Rat

The purpose of this study was to evaluate the effects of thyroid hormone (3,3′,5-triiodo-L-thyronine) on cell proliferation and cell death in the periodontal ligament of rat. Twenty-four adult male Sprague- Dawley rats, weighing 400-500g, were used in this study. The animals were divided into 3 groups; control group (n=4), normal saline injection group (n=10), and thyroid hormone injection group (n=10). After one-week of adaptation period, the control group received the intraperitoneal injection of normal saline every morning for 7 days with the amount of 1ml/kg body weight, and for the experimental group, of 3,3′,5-triiodo-L-thyronine (T3, T63971TM, Sigma- Aldrich Korea LTD) every morning for 7 days with the amount of 100 ㎍/kg body weight. The rats were sacrificed for histological study. Each sample was divided into two specimens; right and left incisors. One incisor was sectioned along the long axis and the other incisor was cross sectioned near the apex. Sections were collected onto silane coated glass slides at a thickness of 5㎛. The sections were stained with hematoxylin-eosin. Additional immunohistochemical staining procedures of proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) were carried out. Light microscopic images were photographed, analyzed and compared. In microscopic examination with

H-E stain, T3 injection group showed the typical histologic feature. There were a lot of congested blood vessels in the periodontal tissue. The cells of periodontal tissue showed abundant new bone formation compared those in the control group. In pulp, the number of blood vessel was increased in the same area of magnification and each blood vessel was highly congested.

The data were analyzed statistically with Kruskal-Wallis 1-way Anova with the significance level at $p<0.01$. To compare between groups, Mann-Whitney U-Wilcoxon Rank Sum W Test was used. Data were expressed as median and range. The number of PCNA-positive cell in the T3 injection group was significantly higher than those of control group and those of normal saline injection group ($p<0.01$). There was no difference with the number of PCNA-positive cell between in control group and in normal saline injection group ($p>0.01$). In every groups, TUNEL-positive cells were only rarely found. There was no specificity of the distribution of the TUNEL-positive cells in every group. No statistically significant differences were noted among control group, normal saline injection group and T3 injection group ($p>0.01$).

Systemic T3 injection enhanced the turnover rate of cells in the periodontal ligament without any other external stimulation and may cause to increase the eruption rate of tooth in rat.

Key words : thyroid hormone, PCNA, TUNEL, rat, cell proliferation, cell death

The Effect of Thyroid Hormone on the Cell Proliferation of Periodontal Ligament in Rat

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I. Introduction

Thyroid hormones play an important role in normal growth and development in growing animals, increasing the basal metabolic rate on cellular level. They affect not only the soft tissue but also the hard tissue. Especially, thyroid hormones influence the cellular turnover rate that leads to an increased rate of cellular proliferation and death (Mosekilde *et al.,* 1990, Tapp, 1966).

It has been reported that thyroid hormone may control the rate of tooth movement and it can reduce the amount of root resorption during the orthodontic treatment (Christiansen, 1994). Kim (2001) reported that a hyperthyroidism patient showed remarkably rapid tooth movement who was treated to correct the unerupted upper left canine by forced eruption

procedure. He reported that there would be a close relationship between the eruption rate and the hormonal levels of thyroid. From the controlled animal study, he concluded that the thyroid hormone may increase the eruption rate of tooth.

Periodontal ligament exists between two mineralized connective tissues, cementum and alveolar bone, and it is a complex, highly vascular and cellular connective tissue. Lekic *et al.* (1997) reported that periodontal ligament has the complex of cells that are progenitors for cementoblasts, osteoblasts and fibroblasts. Periodontal ligament has important functions to maintain the tooth and to transmit the occlusal force to alveolar bone (Schroeder, 1986). And it is claimed that it plays an important role in tooth eruption. It may have a close relationship between the histological changes in periodontal ligament and the eruption rate of tooth.

This study aimed to evaluate the effects of thyroid hormone on periodontal ligament without any other external stimulation. Immunohistochemical stains with proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) were used to detect cell proliferation and cell death in the periodontal ligament.

Ⅱ. Material and methods

1. Animals

Twenty-four adult male Sprague-Dawley rats, weighing 400-500g each, were used in this study. The animals were supplied from Go-Ma Bio tech Co, KOREA.

Rats were cared in SPF barrier area of Department of Laboratory Animal Medicine, College of Medicine, Yonsei University. The temperature (22℃) and humidity (55%) were maintained constantly and illumination was controlled under 12-hours light-dark cycle. The diet (Cheil Jedang Co. USA) and the water (RO water) were supplied *ad libitum.* Rats were acclimated for one week before the experiments. The care of laboratory animals in these experiments were based on the Guidelines and Regulations for Use and Care of Animals in Yonsei University.

2. Preparation of tissues

The animals were divided into 3 groups; control group (n=4), normal saline injection group (n=10), and thyroid hormone injection group (n=10). All animals were given one-week of adaptation period before experiment. In control group, each rat was weighed and sacrificed for

histological study. The rats of normal saline injection group were weighed and received the intraperitoneal injection of normal saline every morning for 7 days with the amount of 1ml/kg body weight. Last day of a week, rats were weighed again and sacrificed for histologic study. The rats of last group were weighed and received the intraperitoneal injection of 3,3′,5-triiodo- L-thyronine (T3, T63971™, Sigma-Aldrich Korea LTD) with the amount of 100㎍/kg body weight. Same dose of T3 was administered based on the previous study which was effective in increasing eruption rate of teeth in rats (Kim, 2001). The injection was done every morning for 7 days. Last day of a week, rats were weighed again and sacrificed for histological study. Mandibles were removed and stored in 4% paraformaldehyde in 0.2M phosphate buffer (pH7.3) at 4℃ for 2days. The specimens were demineralized in 10% EDTA, then were rinsed with 0.2M phosphate buffered saline (PBS), dehydrated with graded ethanol and embedded in paraffin. Each sample was divided into two specimens; right and left incisors. One incisor was sectioned along the long axis and the other incisor was cross-sectioned near the apex. Sections were collected onto the silane coated glass slides at a thickness of 5㎛ approximately. The sections were stained with hematoxylin-eosin for basic histological examination. Immunohistochemical staining procedures with proliferating cell nuclear antigen (PCNA) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) were carried out. Light microscopic images were photographed, analyzed and compared.

The method of Immunohistochemical stains with a PCNA monoclonal antibody (diluted at 1 : 100; DAKO, Denmark) was conducted as follows. Sections were deparaffinized with xylene, and washed with 100% alcohol and PBST(phosphate buffered solution+tween 20). Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 in methanol for 10minutes. To prevent non-specific binding, the sections were incubated with 10% serum for 10minutes in a 100% humidity chamber. PCNA antibody was incubated overnight at 37℃. Sections were washed in PBST and incubated with biotinylated anti-mouse/anti-rabbit IgG(H+L) for 10minutes at room temperature in a humidity chamber. After washed in PBST three times for 5 minutes, sections were incubated with horseradish peroxidase streptavidin for 10minutes at room temperature in a humidity chamber. Sections were stained with 3,3′- diaminobenzidine for 5minutes, washed in distilled water, counterstained in hematoxylin. After dehydration with 70, 90, and 100% alcohol, coverglass was slipped over each section. As a positive control, the epithelial cell of the oral mucosa were observed in the same section.

TUNEL method was done as follows, the sections were deparaffinized with xylene, rehydrated with 100% alcohol and PBST. After incubated with 0.1M Tris-HCl pH7.5 for 30minutes at 15-25℃, sections were incubated with 16.2μg/ml proteinase K solution (in 10mM Tris-HCl buffer, pH 7.4) for 15-30minutes at 21-37℃ and then were incubated with 3% H_2O_2 in methanol for 10minutes at room temperature to block

endogenous peroxidase activity. After washed with PBST, In Situ Cell Death Detection Kit, POD (Roche, Germany) was used. Each sections were incubated with 50㎕ TUNEL reaction mixture for 60minutes at 37℃ and washed three times with PBST. The sections were added with 50㎕ converter-POD and incubated 30minutes at 37℃. And then 50-100㎕ DAB substrate were added on the sections for 10minutes at 15-25℃. Prior to analyze with light microscope, the sections were counterstained with hematoxylin. Negative and positive controls were included in each experimental set up. A negative control section was incubated with 50㎕ Label solution without terminal transferase, instead of TUNEL reaction mixture. A positive control section was incubated with DNase I, grade I (3U/ml in 50mM Tris-HCl, pH 7.5, 10mM $MgCl_2$ 1mg/ml BSA) for 10minutes at 15-25℃ to induce DNA strand breaks, prior to labeling procedures.

3. The counting of PCNA- or TUNEL-positive cells

PCNA-positive cells and TUNEL-positive cells were counted within a unit area (300㎛^{2}) under magnified(x200) light microscopic view. To enhance the objectivity, the number of positive cells in each section was counted at three different portions and obtained median and range.

4. Statistical analysis

The data were tested statistically with Kruskal-Wallis 1-way Anova with the level of significance at $p<0.01$.

Mann-Whitney U-Wilcoxon Rank Sum W Test were used to compare between groups. Data were summarized as median and range.

Ⅲ. Result

1. Histologic findings

The result showed that the control group had a regular arrangement of periodontal fibroblasts and blood vessels. No histological difference between the control group and the normal saline injection group was found (Fig 1). In contrast to control group and normal saline injection group, T3 injection group showed typically different microscopic views. Many congested blood vessels were found along the periodontal ligament. The cells of periodontal tissue exhibited remarkably hyperchromatic nuclei. There showed enhanced bone remodeling activities in contrast to that of control group (Fig 2). In pulp, the number and size of blood vessels were increased and all blood vessels were highly congested with RBC (Fig 3).

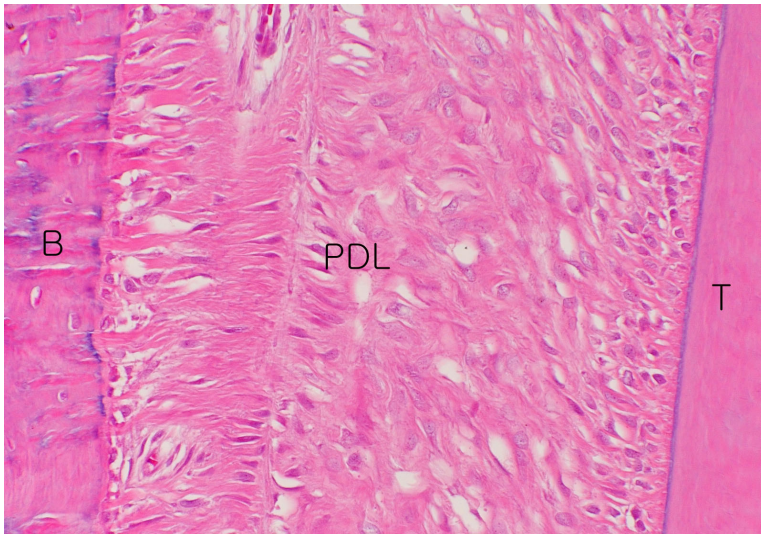

Fig 1. The microscopic view of periodontal tissue in the control group of a rat. Periodontal fibroblasts were well-aligned. PDL (periodontal ligament), B (alveolar bone), T (Tooth) / magnification×200, H-E stain.

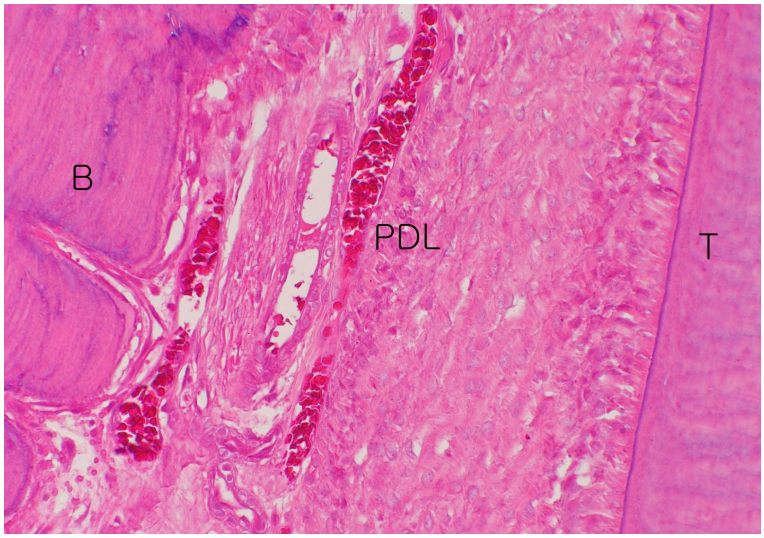

Fig 2. The microscopic view of periodontal tissue in the T3 injection group of a rat. Congested blood vessels and active bony remodeling activity were found.PDL (periodontal ligament), B (alveolar bone), T (Tooth) / magnification×200, H-E stain.

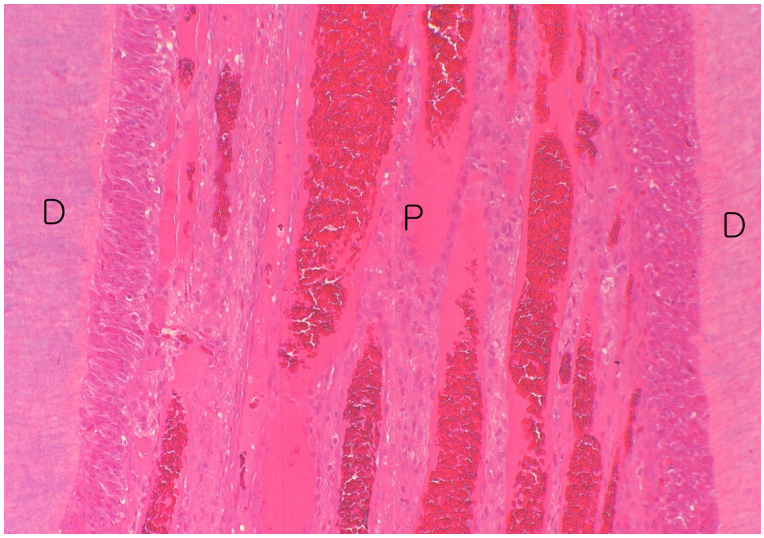

Fig 3. The Magnified view of pulpal tissue in the T3 injection group of a rat. The number and size of blood vessels are increased and blood vessels are highly congested. D(dentin), P(pulp) / magnification×100, H-E stain.

2. PCNA-positive cells

The nuclei of periodontal ligament cells were immunohistochemically stained with PCNA. PCNA-positive cells were found near the alveolar bone and the root surface compared with the middle portion of the periodontal ligament in all groups (Fig 4,5,6). The number of PCNA-positive cells in T3 injection group was significantly higher than those in control group and normal saline injection group ($p<0.01$)(Fig 7). There was no statistically significant difference between the control group and the normal saline injection group ($p>0.01$) (Table 1).

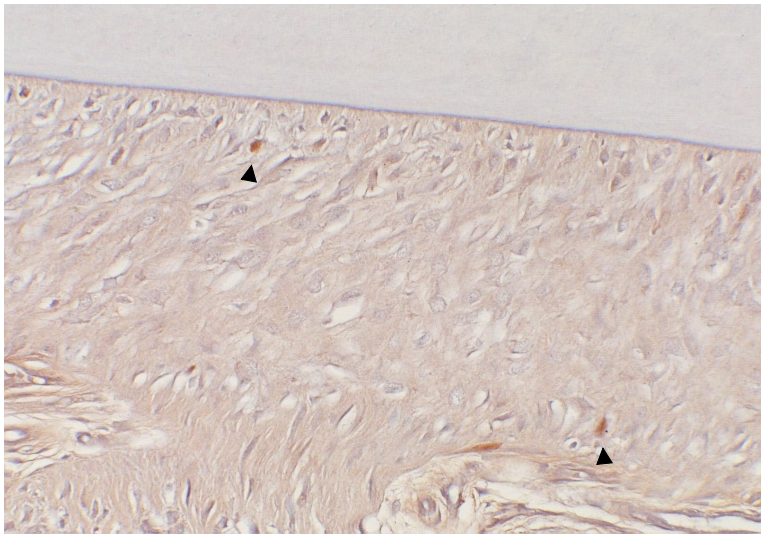

Fig 4. PCNA-positive cells (arrow heads) in the periodontal ligament of the control group (X 200). PCNA-positive cells are found near the alveolar bone and the root surface compared with the middle portion of the periodontal ligament in all groups.

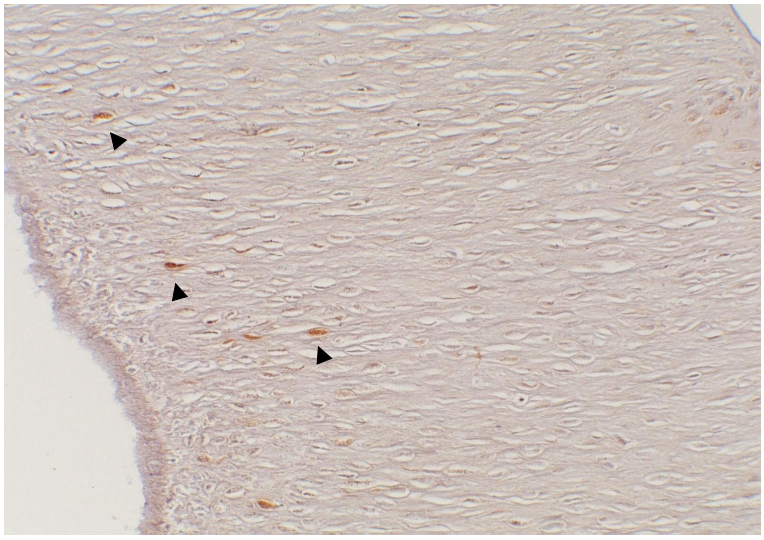

Fig 5. PCNA-positive cells (arrow heads) in the periodontal ligament of the normal saline injection group (X 200).

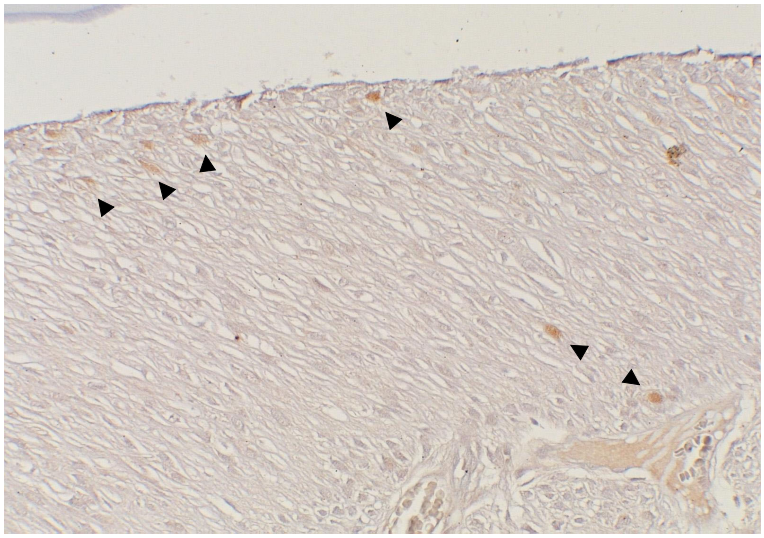

Fig 6. PCNA-positive cells (arrow heads) in the periodontal ligament of the T3 injection group (X 200). PCNA-positive cells are more frequently observed than other groups.

Table 1. Median and range of PCNA-positive cells

	median	minimum	maximum
Control group	4	3	7
Normal saline group	6	2	9
T3 injection group	14.5	5	35

3. TUNEL - positive cells

In every groups, TUNEL-positive cells were only rarely found. There found no specificity of the TUNEL-positive cell distribution in each group (Fig 8, 9, 10). TUNEL-positive cells were observed as condensation and aggregation of nuclear chromatin. No statistically significant differences in TUNEL preparation was noted among control group, normal saline injection group and T3 injection group ($p>0.01$) (Fig 7) (Table 2).

Table 2. Median and range of TUNEL-positive cells

	median	minimum	maximum
Control group	2	1	4
Normal saline group	2	0	8
T3 injection group	3	0	7

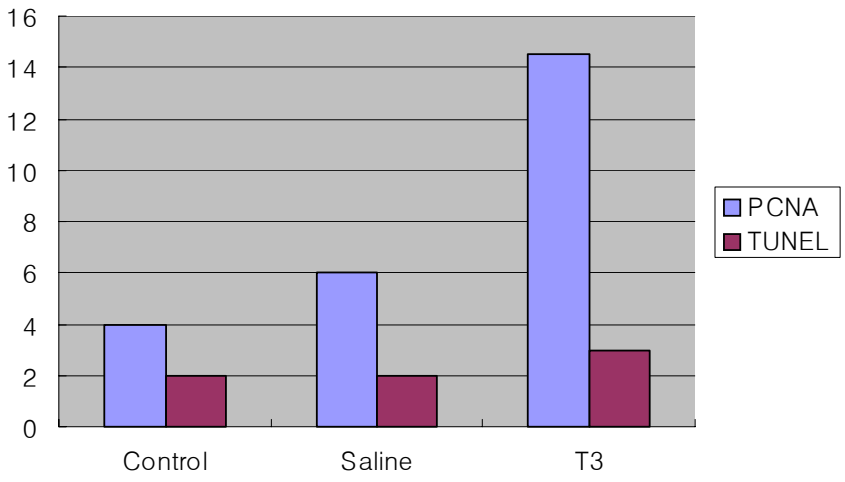

Fig 7. The diagram of PCNA-positive cells and TUNEL-positive cells.

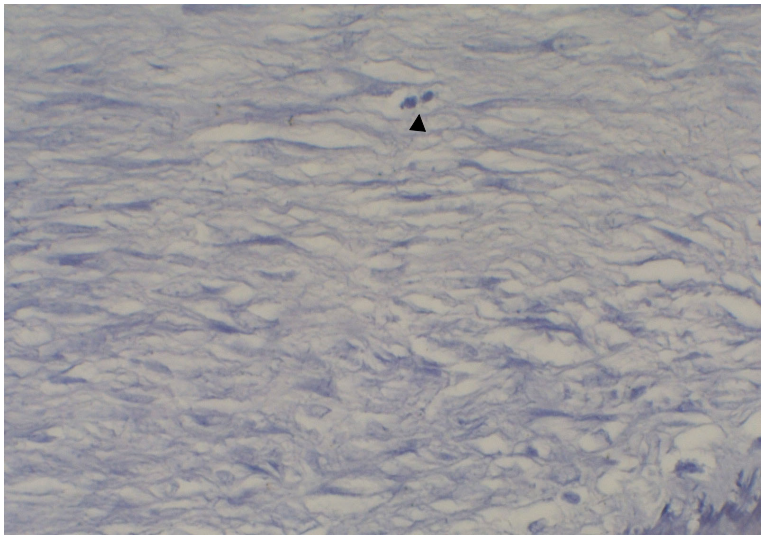

Fig 8. TUNEL-positive cells (arrow heads) in the periodontal ligament of the control group (X 400). TUNEL-positive cells are observed as condensation and aggregation.

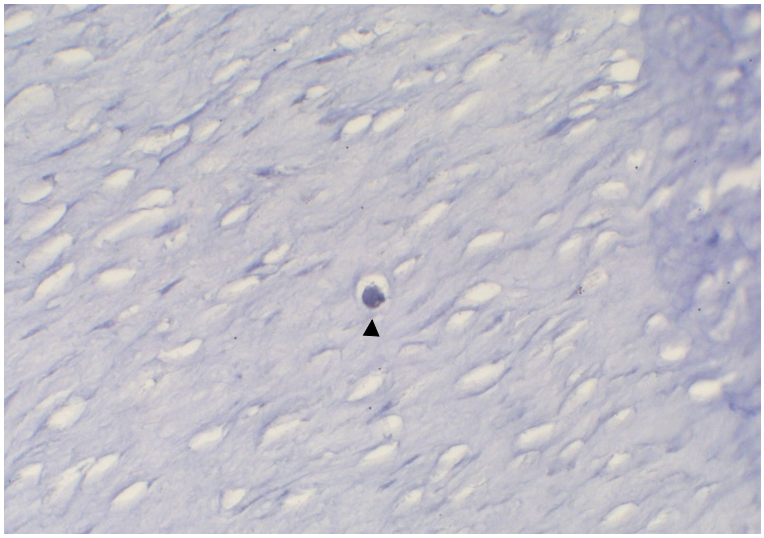

Fig 9. TUNEL-positive cells (arrow heads) in the periodontal ligament of the normal saline injection group (X 400).

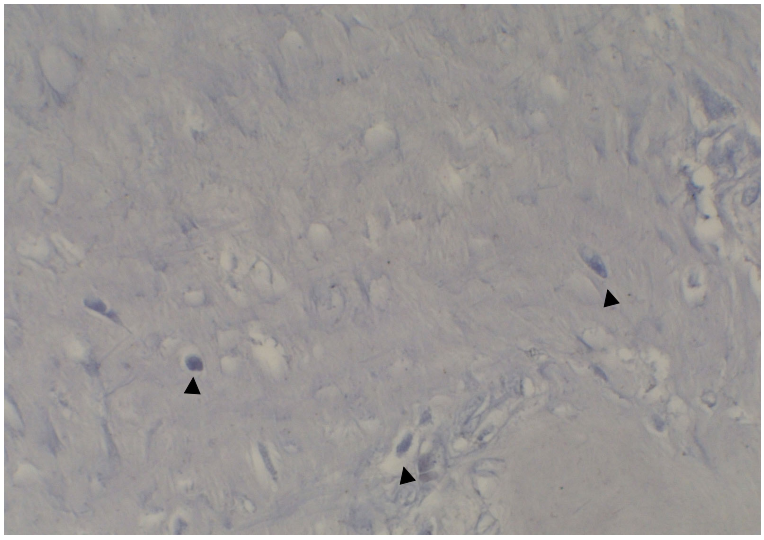

Fig 10. TUNEL-positive cells (arrow heads) in the periodontal ligament of the T3 injection group (X 400). TUNEL-positive cells are observed as small, roughly spherical or ovoid aggregation of nuclear chromatin.

4. Body weight change

All animals were weighed before and after an experimental period of one week injection. Control group and normal saline injection group showed an increase in weight, but in T3 injection group, body weight decreased (Table 3).

Table 3. Changes of body weight between each groups

	Control group		Normal saline group		T3 injection group	
	Mean(gm)	S.D.*	Mean(gm)	S.D.	Mean(gm)	S.D.
Before	441.5	12.07	436.3	13.45	477.5	13.56
After	452	10.10	445.2	13.42	447.7	11.80
Change	10.5	3.70	8.9	1.37	-29.8	4.08

* standard deviation.

Ⅳ. Discussion

Thyroid hormones have negative interactions with sex hormones and growth hormones composing the complex negative feedback system with hypothalamus and pituitary (Mosekidle *et al.* 1990). Thus, in this study, adult male rats were used to avoid other hormonal interaction, and the rats were fed *ad libitum* and given enough space in a cage in order to decrease the stress. During the intraperitoneal injection, rats were anesthetized with ether to reduce the stress to be taken. Nevertheless, the body weight of the T3 injection group decreased. It may be the effect of thyroid hormone by the increased basal metabolic rate.

In contrast to control group and normal saline injection group, T3 injection group showed the marked congested blood vessels in the periodontal ligament and pulp tissue in H-E stain. Kim (2001) reported the same observations as this study. In T3 injection group, abundant osteoid tissue was observed near the alveolar bone, suggesting thyroid hormone stimulated both osteoblastic and osteoclastic activity. According to Christiansen (1994), thyroid hormone enhanced the rate of alveolar bone resorption and decreased root resorption.

It is known that PCNA is a specific marker for cell proliferation. Mabuchi *et al.* (2002) reported that PCNA-positive cells were found near the alveolar bone and the root surface rather the middle portion of periodontal ligament. It was a consistent finding in this study. Mabuchi *et al.* (2002), Baumrind and Buck (1970), Toto and Borg (1968), Jansen

and Toto (1968), Weiss *et al.* (1968), Toto and Kwan (1970), Roberts (1975), Yee *et al.* (1976), Yee and Kimmel (1976) reported that the labeling index of DNA-synthesis is 1-4% in the normally functioning mouse and rat molar ligament. In the control group of this study, the positive ratio to PCNA was approximately 3-4% which is consistent with the previous studies.

Toto and Borg (1968) reported that the periodontal ligament in old mice had both reduced cell population and less capacity for regenerating cells than young mice, and that longer time was required for cells to double in number. According to Toto and Kwan (1970), the distribution of labeled cells showed irregularity because of differences in the thickness of sections and a failure of adequate antibody attachment, which results in a failure of adequate staining. In this study, adult male rats, approximately 20-week-old, were used to avoid the other hormonal influences such as growth hormones and sex hormones. Therefore, it may be explained that the PCNA-positive cells in this study were reduced in number and were not evenly distributed. When staining the sections, the incubation time of primary antibody and temperature need to be modified.

Mabuchi *et al.* (2002), and Gould *et al.* (1977, 1980) claimed that stimulation of the periodontal ligament of mouse by traumatic wounding or orthodontic force increases the labeling index. In this study, PCNA-positive cells in the periodontal ligament of mandibular incisor of rat increased only by the intraperitoneal injection of 3,3′,5-triiodo-L-thyronine (T3, T63971™, Sigma-Aldrich Korea LTD) with the

amount of 100μg/kg body weight without any other external stimulation. As thyroid hormone is considered in the complex feedback system, small amount of exogenous T3 did not increase circulating thyroxine levels. So large dose of T3 (about 3 times higher than the average level) was injected every morning. Many PCNA-positive osteoblasts were observed near the alveolar bone. It means that thyroid hormone enhances the turnover rate of cells in periodontal ligament.

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological, biochemical, and molecular changes of dying cells. Apoptosis is the most common form of eukaryotic cell death. TUNEL is an immunohistochemical staining method to detect apoptotic cell death of a single cell level. This method is based on the specific binding effect of TdT to 3′-OH of DNA. Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single breaks in high molecular weight DNA. Labeling free 3′-OH termini with modification in an enzymatic reaction can identify those broken DNA strands. Incorporated fluorescein is detected by anti-fluorescein antibody Fab fragments. After substrate reaction, stained cells could be analyzed under light microscope. Gavrieli *et al.* (1992) revealed that the fragments of DNA strands are prominent in cells, which have high proliferative or metabolic activity.

It is conceivable that the balance between cell proliferation and cell death can be maintained in the periodontal ligament to maintain regular

space. But in this study, the number of TUNEL-positive cell was not as many as that of PCNA-positive cell. Coles *et al.* (1993) suggested that even large-scale cell death may be easily missed in sections and only a few apoptotic cells are found in any single section. For example, it has been estimated that more than 97% of newly formed thymocytes die during normal thymus development, but that only 0.2% of these cells are evident in tissue sections. Apoptosis of each cell occurs relatively short period of time considering the total period of large-scale cell death. This was consistent with the findings in this study that only few TUNEL-positive cells could be detected, suggesting that the periodontal ligament may regulate the number of cells to maintain homeostatic functions. Thus, in this study, it was assumed that the proliferating cells may undergo cell death, but only few cells were detected. In T3 injection group, more TUNEL-positive cells were detected than in other groups, but there was no statistical significance.

In T3 injection group, there were increased cell proliferation than in other groups. The increased cellular turnover rate may caused the increased eruption rate of tooth. These results may support Kim (2001)'s suggestion that increased level of serum thyroid hormone increases eruption rate of lower incisor in rats.

Ⅴ. Conclusion

To evaluate the effect of thyroid hormone (3,3′,5-triiodo-L-thyronine) on the rate of cell proliferation and cell death in the periodontal ligament in rat, twenty-four adult male Sprague-Dawley rats, weighing 400-500g, were used in this study. The results are summarized as;

1. In T3 injection group, the number of PCNA-positive cell increased with statistical significance ($p<0.01$). There was no statistically significant difference with the number of PCNA-positive cell between control group and normal saline injection group.

2. TUNEL-positive cells were rarely detected in all groups and no statistically significant difference was shown between the T3 injection group and the other groups.

3. Systemic T3 injection raised the turnover rate of cells in the periodontal ligament without any other external stimulation and may cause to increase the eruption rate of tooth in rat.

From these results, we have a possibility to help the patient who has developmental problems and eruption disturbances of teeth. But further studies on dosage of thyroid hormone, duration of administration and side effects are needed to apply thyroid hormone for clinical use.

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국문요약

갑상선 호르몬이 흰쥐의 치주 인대 세포의 증식에 미치는 영향

연세대학교 대학원 치의학과

박 동 석

지도교수 : 손 홍 규

이 연구의 목적은 갑상선 호르몬이 흰쥐의 치주 인대 세포의 증식과 사멸에 미치는 영향을 분석하기 위함이다. 400-500g 사이의 수컷 흰쥐(Sprague-Dawley rats) 24마리를 사용하였다. 실험동물은 3 집단으로 분류하였다; 정상 대조군 4마리, 생리식염수 투여군 10마리, 갑상선 호르몬 투여 실험군 10마리. 1주일간의 적응기간 후 생리식염수 주사군은 체중 당 1ml/kg의 생리식염수를 1주일간 복강 투여하였다. T3 실험군은 체중 당 100㎍/kg의 3,3′,5-triiodo-L-thyronine (T3, T63971TM, Sigma-Aldrich Korea LTD)를 1주일간 복강 투여 하였다. 1주일 후 실험동물을 희생시켜 조직학적 분석 및 비교를 시행하였다. 하악 중절치의 치주 인대를 H-E 염색과 면역학적 염색 방법인 PCNA와 TUNEL 방법으로 관찰하였다. 조직학적 소견 상 T3투여군의 치주인대에서는 상대적으로 울혈된 혈관들이 다수 관찰되었다. 또한 새로운 골 형성도 더 많이 관찰되었다. 치수내에서도 많은 혈관의 증식과 울혈이 관찰되었다.

통계학적 분석을 위해 Kruskal-Wallis 1-way Anova 방법을 사용하였고

각 군당 비교를 위하여 Mann-Whitney U-Wilcoxon Rank Sum W Test 방법을 사용하였다.

T3 실험군에서 PCNA-positive 세포의 수는 정상 대조군과 생리식염수 투여군과 비교하여 통계학적 유의차 있게 높게 나타났다 ($p<0.01$). 정상 대조군과 생리식염수 투여군간에는 통계학적 유의차가 없었다 ($p>0.01$).

모든 군에서 TUNEL positive 세포는 매우 드물게 발견되었다. 각 군간 통계학적 유의차는 없었다 ($p>0.01$).

전신적 T3 호르몬의 투여는 다른 외부자극 없이 치주 인대 세포의 turn over rate을 증가시켰고 치아 맹출 속도를 증가시키는데 기여한다.

Key Word : 갑상선 호르몬, PCNA, TUNEL, 흰쥐, 세포증식, 세포사멸.