치의학석사학위논문

Effects of Resveratrol, Quercetin and Curcumin on Human Dental Stem Cells

Resveratrol, Quercetin, Curcumin이 사람 치아 줄기세포에 미치는 영향

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서울대학교 치의학대학원
치의학과
김 민 수

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지도교수 정 필 훈

이 논문을 치의학석사 학위논문으로 제출함

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치의학과

김 민 수

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위 원 장 김 현 만 (인)

부 위 원 장 정 필 훈 (인)

위 원 이 승 복 (인)

Abstract

Effects of Resveratrol, Quercetin and Curcumin on Human Dental Stem Cells

Kim Minsu

Department of Dentistry, School of Dentistry

The Graduate School

Seoul National University

The biological effects of plant-derived compounds, resveratrol, quercetin and curcumin, have been reported, but their effects on human dental stem cells have not been studied. These three plant-derived compounds have similar chemical structures, polyphenols. There are many researches about these compounds having similar biological activities related colon cancer (by regulating Wnt-signaling pathway) and other chronic diseases. And they have been reported to promote proliferation of hMSCs, or to suppress proliferation of human cancer cells. These results were

thought to be controversial, but to contrary, they might have the broad usages by the diversities of biological effects on the cells. Also, resveratrol, quercetin and curcumin have been reported to promote mineralization of hMSCs, but the doses for mineralization of hMSCs were not defined yet.

Human dental stem cells seem to be promising sources of dental regeneration, biomaterials affecting their activities and functions are still researched. There are many candidating biomaterials for regenerative therapy, but still doubtful.

Therefore, in this study through a view point of bio-informatics based on the chemical structures, polyphenols thought to be safe and effective biomaterials for dental regeneration, at least for proliferation and mineralization of human dental stem cells including periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs). We studied three natural phenols including resveratrol, quercetin and curcumin, and tried to figure out the relationship between doses of polyphenols and cellular effects on the hDSCs.

The proliferation of human dental stem cells was studied with MTT assay at 24, 48, 72 and 96 hours, and the mineralization of hDSCs was assessed by Alizarin red S staining for 2 and 3 weeks. Relative gene expression levels were analyzed by means of real-time PCR.

The experimental groups treated with resveratrol, quercetin and curcumin at low concentration, showed increased proliferation of

hDSCs. But at high concentration, they showed suppression of proliferation or cytotoxic effects on hDSCs, especially on PDLSCs. The results of Alizarin red S staining showed resveratrol, quercetin and curcumin increased mineralization of hDSCs at low concentrations. The results of real-time PCR supported the mineralization effects of these plant-derived compounds on hDSCs.

Our results indicate that the safe and effective range of concentrations for regenerative dentistry might be 1 μM to 10 μM. Resveratrol showed most promoted proliferation on hDSCs at 1 μM concentration, quercetin and resveratrol showed most promoted mineralization of DPSCs and PDLSCs at 5 μM concentration respectively. And these three compounds inhibited proliferation of hDSCs induced by which thought to be an apoptosis under the concentrations over 50 μM, especially with quercetin and curcumin. These results were also consistent with the reports that resveratrol, quercetin and curcumin could be the related bio-materials for the cancer research. And they could be the useful natural compounds related for the regeneration using dental stem cells

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keywords: Human dental stem cells, resveratrol, quercetin, curcumin, proliferation, mineralization

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I. Introduction

The periodontium is composed of several supporting tissues such as root cementum, periodontal ligament (PDL), alveolar bone and the gingival tissues adjacent the tooth[1]. Among them, the cementum has been known as having important parts of regeneration of periodontium[2]. But still, there are some facing problems in researches on regenerative dentistry using human dental stem cells.

1) Lack of embryological approaches in cementogenesis.

2) Limited to be focused on the bone regeneration, not periodontium including cementum.

3) Lack of cementum specific marker, which results in investigating cementogenic materials. So, to find a basic therapeutic method for whole regeneration of the periodontium, it must be investigated which candidating materials having positive effects on bone or cementum formation with human dental stem cells first, and the molecular mechanisms.

Recently, interests of many kinds of natural plant-derived compounds get increased by researchers, clinicians and public for prevention and treatment for the varying chronic diseases[3]. It has been known that the use of natural plant-derived compounds decreases the pathologic conditions, related with cancer, nervous system disorders, cardiovascular disorders and inflammation[4,5].

Plants contain varying effective materials which can improve the resistance against cellular stresses and cytotoxicities. Among them, natural phenols such as resveratrol (non-flavonoids), quercetin and curcumin have gained much attention for their abilities[6]. There are many kinds of natural phenols, including flavonoids, isoflavonoids, stilbenoids, diarlyheptanoids, xanthonoids and coumarins. They have broad spectrum of chemical, physical and biological properties, but they can be classified according to their similar chemical structures with phenols, and can be found in nature commonly.

Resveratrol is a polyphenolic phytoestrogen or one of the stilbenoid (trans-3,5, 4′-trihydroxysilbene; Figure 1A) which can be collected from red grapes, vines, some other fruits and the roots of *Polygonum cuspidatum*[7]. And it is known to be produced by some plants against the injuries or attacks from the pathogens. Resveratrol is also known as acting on the estrogen receptors[8], binding to ERs, so it has biological effects on the cardiovascular system and bone formation[9]. Many other biological effects of resveratrol have been reported, including cardiovascular protection[10], anticancer activity[11] and positive effects on proliferation and differentiation in human and mouse mesenchymal stem cells (MSCs)[12,13]. However, its effects on human dental stem cells (hDSCs) have not been reported.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl-1,6-heptadiene-3,5-dione); Figure 1C) is a yellowish compound, one of the diarylh

eptanoids which can be found from the roots of *Curcuma longa Linn*, the Zingerberaceae family[14]. Curcumin has been reported to have positive effects on the inflammatory status of organisms, m utations and cancers[15,16]. And there are many reports about curcu min having effects on the bone and fat formation[17-20]. However, it s effects on hDSCs have not been reported.

Quercetin (3,3′,4′,5,7-Pentahydroxyflavone;2-(3,4-Dihydroxypheny l)-3,5,7-trihydroxy-4H-chromen-4-one; Figure 1B) is a flavonoid which can be found in onions, and fruits like apples or grapes[21]. It has been known that it has anti-inflammatory[22-24], cytoprotectiv e[25-27] and DNA-protective effects[28,29]. But the effects of quercetin on the bone formation are still controversial[30-33].

Human dental stem cells (hDSCs) are rising sources of the re- generative dentistry[34,35]. Thus investigation on these cells are im- portant ways on the dental regeneration. There are accumulating evidences suggesting natural phenols are the important materials for the proliferation and differentiation of mesenchymal stem cells[36]. As stated above, these three natural phenols have been reported to promote proliferation of hMSCs, or to suppress pro- liferation of human cancer cells. These results were thought to be controversial, but to contrary, they might have the broad spectrum of effects on the cells. Also, resveratrol, quercetin and curcumin have been reported to promote mineralization of hMSCs, but the doses for mineralization of hMSCs were not de- fined yet.

Human dental stem cells seem to be promising sources of dental regenerations, biomaterials affecting their activities and functions are still researched. There are many candidating biomaterials for regenerative therapy, but still doubtful.

So the aim of this study is to investigate the effects of plants-derived compounds (resveratrol, curcumin and quercetin) on proliferation and mineralization of hDSCs including dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs).

Based on bio-informatics these three compounds thought to have positive effects on proliferation and differentiation on hDSCs, which might be an important bio-molecules related with dental regeneration.

II. Materials and Methods

Primary cell culture from human dental tissue

Human third molars or premolars were collected from healthy young patients who underwent tooth extraction, under the protocol approved by the Institutional Review Board of Seoul National University Dental Hospital, Seoul Korea (IRB 05004). PDL tissues were separated from the root surface carefully by surgical blades to obtain human PDLSCs. In order to isolate DPSCs, tooth were cut around the cemento-enamel junction (CEJ) with sterilized dental fissure burs and separated the pulp tissues from the pulp chamber and canals by barbed broaches.

Separated tissues were digested in solution of 3 mg/mL collagenase type I (worthington Biochem, Freehold, NJ) and 4 mg/mL dispase (Boehringer, Mannheim, Germany) for 1 hour at 37℃ incubator. Single-cell suspensions were performed by passing the cells through a 40 μm strainer (BD Labware, Frakin Lakes, NJ) and cultured in normal growth medium with 5% PRF extract (PRFe)[37] and incubated at 37℃ with 5% CO2.

Plants-derived compounds

Resveratrol, quercetin and curcumin (Sigma Aldrich, St. Louis, MO) were purchased as solid or powder types, and dissolved in dimethylsulfoxide (DSMO) as concentration of 10 mM. These compounds were diluted as 0.01 to 0.0001 for treatment on the hDSCs. (DMSO were contained less than 1% v/v)

Resveratrol and curcumin were stored at -20℃ refrigerator, and quercetin was stored at room temperature.

Cell proliferation assay (MTT assay)

The proliferation of hDCSs was measured by using the Cell Counting Kit-8 (DOJINDO Laboratories, Japan). PDLCSs and DPSCs (1.0×10^4 cells/well) were seeded in 96-well plates and cultured for 12 hours and medium was changed to growth medium with 5% PRFe (PRF extract). At the same time, various concentrations of resveratrol, curcumin, and quercetin (0, 1.0, 5.0, 10.0, 50.0 and 100 μM) were treated to each well. 10 μl of dye solution (Cell Counting Kit-8, DOJINDO Laboratories, Japan) was added at different time durations such as 24, 48, 72 and 96 hours. The cells were incubated in 5% CO_2 at 37℃ for 4 hours, and then the absorbances were measured with microplate reader (Fluostar Optima, BMG LABTECH, Cary, NC, USA) read at 450 nm. The resulting values were expressed as O.D. value means ± standard deviations.

Mineralization and Alizarin red S staining

For visualizing the mineralization of hDSCs by the plants-derived compounds, Alizarin red S staining was used. DPSCs and PDLSCs (4.0×10^4 cells/well) were seeded in 24-well plates or 12-well plates and cultured under normal growth medium. After 12 hours, 1 μM, 5 μM, and 10 μM concentrations of resveratrol, quercetin and curcumin were treated to experimental groups, and the cells were cultured in differentiation medium with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (Sigma, St. Louis, MO, USA) and 5% PRFe (PRF extract). The differentiation medium was changed every three days and cultured for 2 or 3 weeks. Mineralization nodules were observed and stained by 2% Alizarin red S staining solution (pH 4.2) (Sigma, St. Louis, MO, USA).

RNA preparation and real-time PCR

After 2 weeks (PDLSCs) or 3 weeks (DPSCs) of differentiation, the RNAs were extracted from the cells by Trizol reagent (INvitrogen, Carlsbad, CA, USA) based on the manufacturers's protocol. All the RNA was quantitatively analyzed by NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNA was synthesized by rel-time PCR, and primer sets for re-al-time PCR were prepared by using Hypoxanthine-guanine phosphoribosyltransferase (HPRT), Collagen I (Col I), Osteopontin (OPN), Osteocalcin (OCN) and Runt-related transcription factor 2 (Runx2). Real-time PCR was performed using SYBR green PCR Master Mix (Applied Biosystem, Warshinton, Cheshire, United Kongdom). Manufacturer's protocol was used for real-time PCR. Oligonucleotide primer-sequences were shown in table 1. Relative expression levels were analyzed by the $\Delta\Delta C_t$ method[38].

III. Results

The effects of plant-derived compounds on proliferation of human dental stem cells. (MTT assay)

The effects of resveratrol, quercetin and curcumin on proliferation of DPSCs

The effects of proliferation on DPSCs in various concentrations of resveratrol, quercetin and curcumin were shown in Figure 2A, 3A and 4A. The O.D. values were measured at 24, 48, 72, and 96 hours (n=4).

The proliferation of DPSCs was increased at low concentrations, 1 μM and 5 μM of resveratrol after 72 hours (Fig. 2A). And DPSCs treated by resveratrol showed most increased proliferation among these three compounds. DPSCs treated by quercetin showed slightly increased proliferation compared to the control group, but at these low concentrations, 1 μM, 5 μM and 10 μM, there was no signigicant difference (Fig. 3A). In case of DPSCs treated by curcumin showed weakest effects on proliferation, they showed almost same levels of proliferation compared to the control group after 72 hours at low concentrations (Fig. 4A).

However, DPSCs treated by these three compounds showed de-

creased proliferation at higher concentrations than 10 μM after 48 hours (Fig. 2A, 3A, 4A). And this decreased proliferation well showed in DPSCs treated by higher than 50 μM curcumin after 48 hours of culturing (Fig. 4A). In case of DPSCs treated by resveratrol and quercetin, they showed decreased proliferation at high concentrations, but they showed less decreased effects than DPSCs treated by curcumin. Especially, quercetin showed almost same levels of proliferation to the 10 μM concentration compared with the control group (Fig. 3A).

The effects of resveratrol, quercetin and curcumin on proliferation of PDLSCs

The effects of proliferation on PDLSCs in various concentrations of resveratrol, quercetin and curcumin were shown in Figure 2B, 3B and 4B. The O.D. values were measured at 24, 48, 72, and 96 hours (n=4).

The proliferation of PDLSCs was also increased at low concentration, 1 μM resveratrol after 96 hours (Fig. 2B). But PDLSCs treated by resveratrol higher than 5 μM concentrations, they showed decreased proliferation compared to the control group (Fig. 2B). PDLSCs treated by quercetin showed almost same results with PDLSCs treated by resveratrol, but decreased proliferation over 50 μM concentrations seemed to be more significant (Fig. 3B). PDLSCs treated by curcumin showed increased proliferation at low concentrations, 1 μM to 10 μM, but they showed decreased proliferation over 50 μM concentrations of curcumin also (Fig. 4B).

According to a microscopic view of the PDLSCs treated by resveratrol, quercetin and curcumin with higher than 50 μM concentrations showed significant suppressions on proliferation, and especially in case of 100 μM concentration of quercetin and curcumin, they showed cytotoxic effects after 48 hours of culturing. It showed in Figure 8.

The effects of plant-derived compounds on Mineralization of human dental stem cells.

The effects of resveratrol, quercetin and curcumin on mineralization of DPSCs after 2 weeks for differentiation

The results of Alizarin red S staining on DPSCs after 2 weeks of differentiation were shown in Figure 9 to 11. The mineralization of DPSCs treated by resveratrol increased at 1 μM and 10 μM concentrations on 14 days (Fig. 9A). But it did not seem to be more significant than in cases of 3 weeks of DPSCs (Fig. 12A) or 2 weeks of PDLSCs (Fig. 9A). According to a microscopic view, they started to show stained mineralization nodules at 2 weeks after differentiation, but the numbers of them seemed to be undistinguishable.

DPSCs treated by quercetin showed increased mineralization after 2 weeks of differentiation with dose dependent manners (Fig. 10A). But they also did not seem to be more significant than in cases of 2 weeks of PDLSCs (Fig. 10A) or 3 weeks of DPSCs (Fig. 13A). But they seemed to start differentiation on mineralization increasingly with increasing concentrations of quercetin, and showed increased mineralization nodules at the same ways (Fig. 10B). DPSCs treated by quercetin showed fastest mineralization and showed most mineralization nodules after 2 weeks of

differentiation (Fig. 10).

DPSCs treated by curcumin showed most increased mineralization at 1 μM concentration of curcumin after 2 weeks of differentiation (Fig. 11A). But according to the microscopic views, they seemed to be just started to show stained mineralization nodules (Fig. 11B). DPSCs treated by curcumin showed slowest mineralization and showed least mineralization nodules compared to DPSCs treated by resveratrol or quercetin after 2 weeks (Fig. 9, 10, 11).

The effects of resveratrol, quercetin and curcumin on mineralization of PDLSCs after 2 weeks for differentiation

The results of Alizarin red S staining on PDLSCs after 2 weeks of differentiation were shown in Figure 9 to 11. The mineralization of PDLSCs treated by resveratrol increased in 1 μM and 5 μM concentrations at 14 days (Fig. 9A). And it seemed to be more significant than in cases of 2 weeks of DPSCs (Fig. 9) compared to the control group. According to a microscopic view, they showed many numbers of stained mineralization nodules at 2 weeks after differentiation at 1 μM and 5 μM concentrations of resveratrol (Fig. 9B).

PDLSCs treated by quercetin showed increased mineralization after 2 weeks of differentiation at 5 μM and 10 μM of quercetin (Fig. 10A). They also seemed to be more significant than 2 weeks of DPSCs (Fig. 10A) compared to the control group.

PDLSCs treated by curcumin showed most increased mineralization at 5 μM concentration of curcumin after 2 weeks of differentiation (Fig. 11). But they showed less numbers of mineralization nodules compared to PDLSCs treated by resveratrol or quercetin after 2 weeks of differentiation (Fig. 9B, 10B, 11B).

The effects of resveratrol, quercetin and curcumin on mineralization of DPSCs after 3 weeks for differentiation

The results of Alizarin red S staining on DPSCs after 3 weeks of differentiation were shown in Figure 12 to 14. The mineralization of DPSCs treated by resveratrol increased with dose dependent manners (Fig. 12A). It seemed to be more significant than 2 weeks of DPSCs (Fig. 9A), but showed less mineralization nodules compared to 3 weeks of PDLSCs (Fig. 12B). As a microscopic view, they showed many numbers of stained mineralization nodules at 3 weeks after differentiation at all of the experimental groups compared to the control group (Fig. 12B).

DPSCs treated by quercetin showed increased mineralization after 3 weeks of differentiation at 1 μM and 5 μM of quercetin (Fig. 13A). And they also seemed to be more significant than 2 weeks of DPSCs (Fig. 10A), but showed less mineralization nodules compared to 3 weeks of PDLSCs (Fig. 13B). DPSCs treated by quercetin showed most numbers and biggest sizes of mineralization nodules compared to DPSCs treated by resveratrol or curcumin after 2 weeks (Fig. 13B). These results were consistent with the results of 2 weeks of differentiation.

DPSCs treated by curcumin showed most increased mineralization at 1 μM and 5 μM concentration of curcumin after 3 weeks of differentiation (Fig. 14A). According to the microscopic views,

they showed many numbers of stained mineralization nodules (Fig. 14B). But DPSCs treated by curcumin showed slowest mineralization and least mineralization nodules compared to DPSCs treated by resveratrol and quercetin after 3 weeks (Fig. 12, 13, 14). And these results were consistent with the results of 2 weeks of differentiation.

The effects of resveratrol, quercetin and curcumin on mineralization of PDLSCs after 3 weeks for differentiation

The results of Alizarin red S staining on PDLSCs after 3 weeks of differentiation were shown in Figure 12 to 14. The mineralization of PDLSCs treated by resveratrol increased with dose dependent manners especially at 10 μM concentration of resveratrol (Fig. 12A). It seemed to be more significant than in case of 3 weeks of DPSCs (Fig. 12A), and showed more mineralization nodules compared to 2 weeks of PDLSCs (Fig. 9B). The differences in the numbers or sizes of mineralization nodules seemed to be less distinguishable among the experimental groups (Fig. 12B). But the differences in the numbers or sizes of mineralization nodules seemed to be more significant among the three materials. PDLSCs treated by resveratrol showed most differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to the PDLSCs treated by quercetin or curcumin after 3 weeks of differentiation.

PDLSCs treated by quercetin showed mostly increased mineralization after 3 weeks of differentiation at 5 μM and 10 μM concentrations of quercetin (Fig. 14A) compared to the control group. They also seemed to be more significant than 3 weeks of DPSCs (Fig. 14A), and showed more mineralization nodules compared to 2 weeks of PDLSCs (Fig. 10B). PDLSCs treated by quercetin

showed more differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to the PDLSCs treated by curcumin, but showed less differences compared to PDLSCs treated by resveratrol after 3 weeks of differentiation.

PDLSCs treated by curcumin showed most increased mineralization at 1 μM, 5 μM and 10 μM concentrations of curcumin after 3 weeks of differentiation (Fig. 14A). In the microscopic views, they showed more numbers of stained mineralization nodules (Fig. 14B) compared to the control group. But PDLSCs treated by curcumin showed less differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to PDLSCs treated by resveratrol or quercetin after 3 weeks of differentiation.

Real-time PCR

Real-time PCR results for the DPSCs treated by resveratrol after 3 weeks of differentiation

The results of Real-time PCR (Polymerase chain reaction) were shown in Figure 15 and 16 (n=3). All of the data, C_t-values were analyzed by $\Delta\Delta C_t$-value methods.

In the case of the DPSCs treated by 10 μM concentration of resveratrol after 3 weeks of differentiation were shown in Figure 15A. Expression of Col I and OCN was slightly increased. But expression of Runxs2 and OPN showed two-fold and three-fold increase respectively(Fig. 15A).

Real-time PCR results for the PDLSCs treated by resveratrol after 2 weeks of differentiation

The results of real-time PCR for the PDLSCs treated by 10 μM concentration of resveratrol after 2 weeks of differentiation were shown in Figure 15B. Expression of Col I and Runx2 was slightly increased, expression of OPN was slightly decreased (Fig. 15B). The relative expression level of OCN in the PDLSCs treated by quercetin after 2 weeks of differentiation was not collected.

Real-time PCR results for the DPSCs treated by quercetin after 3 weeks of differentiation

The results of real-time PCR for the DPSCs treated by 1 μM concentration of quercetin after 3 weeks of differentiation were shown in Figure 16A. Expression of Col I and OCN was slightly increased, expression of Runx2 three-fold increase (Fig. 16A). The relative expression level of OPN in the DPSCs treated by quercetin after 3 weeks of differentiation was not collected.

Real-time PCR results for the DPSCs treated by quercetin after 2 weeks of differentiation

The results of real-time PCR for the PDLSCs treated by 1 μM concentration of quercetin after 2 weeks of differentiation were shown in Figure 16B. Expression of Runx2 was slightly increased but expression of Col I was slightly decreased. Expression of OCN showed three-fold increase (Fig. 16B). The relative expression level of OPN in the PDLSCs treated by quercetin after 2 weeks of differentiation was not collected.

IV. Discussion

The purpose of this study was to determine effects of plant-derived compounds such as resveratrol, quercetin and curcumin on the proliferation and mineralization of hDSCs. These three natural phenols could be chosen through bio-informatics based on their chemical structures. There have been many studies about biological effects of these three compounds on cancer cells or other cell types including fibroblasts derived from PDL or pulp tissues. And the results from those studies indicated different effects on different cell types and different maturation levels of cells. As stated above, resveratrol, quercetin and curcumin showed controversial effects of proliferation on hMSCs or on human cancer cells. And it had not been determined which doses of these polyphenols affecting mineralization of hMSCs.

So this study examined the safety and effectiveness of resveratrol, quercetin and curcumin, and tried to determine the relationship between concentrations and the effects on the proliferation or mineralization of these three compounds. Effects of these compounds were studied on dental regeneration, more specifically, proliferation and mineralization of human dental stem cells (hDSCs) including periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs).

The effects of resveratrol, quercetin and curcumin on proliferation of hDSCs (human dental stem cells)

Stem cell proliferation is one of the most important properties for the development and regeneration of human dental tissues. In proliferation phase, these compounds stimulated proliferation under low concentrations, but inhibited proliferation under high concentrations.

The effects of proliferation on hDSCs in various concentrations of resveratrol, quercetin and curcumin were shown in Figure 2, 3 and 4. The O.D. values were measured at 24, 48, 72, and 96 hours (n=4).

The proliferation of DPSCs was increased at low concentrations, 1 μM and 5 μM of resveratrol after 72 hours (Fig. 2A). And DPSCs treated by resveratrol showed most increased proliferation among these three compounds. DPSCs treated by quercetin showed slightly increased proliferation compared to the control group, but at these low concentrations, 1 μM, 5 μM and 10 μM, there was no signigicant difference (Fig. 3A). In case of DPSCs treated by curcumin showed weakest effects on proliferation, they showed almost same levels of proliferation compared to the control group after 72 hours at low concentrations (Fig. 4A). However, DPSCs treated by these three compounds showed decreased proliferation at higher concentrations than 10 μM after 48 hours (Fig. 2A, 3A, 4A). And this decreased proliferation well showed in DPSCs treated by higher than 50 μM curcumin after

48 hours of culturing (Fig. 4A). In case of DPSCs treated by resveratrol and quercetin, they showed decreased proliferation at high concentrations, but they showed less decreased effects than that of DPSCs treated by curcumin. Especially, quercetin showed almost same levels of proliferation to the 10 μM concentration compared with the control group (Fig. 3A).

The proliferation of PDLSCs was also increased at low concentration, 1 μM resveratrol after 96 hours (Fig. 2B). But PDLSCs treated by resveratrol higher concentrations than 5 μM, they showed decreased proliferation compared to the control group (Fig. 2B). PDLSCs treated by quercetin showed almost same results with PDLSCs treated by resveratrol, but decreased proliferation over 50 μM concentrations seemed to be more significant (Fig. 3B). PDLSCs treated by curcumin showed increased proliferation at low concentrations, 1 μM to 10 μM, but they showed decreased proliferation over 50 μM concentrations of curcumin also (Fig. 4B).

According to a microscopic view of the PDLSCs treated by resveratrol, quercetin and curcumin with higher concentrations than 50 μM showed significant suppressions on proliferation, and especially in case of 100 μM concentration of quercetin and curcumin, they showed cytotoxic effects after 48 hours of culturing. It showed in Figure 8.

Theses compounds have been known to affect a broad range of molecular targets including Wnt/β-catenin[39], Sirt-1[40] and ER sig-

naling[41], PPARγ signaling[42], MAPK and ERK signaling[43,44]. Especially, Wnt signaling has been reported to promote proliferation of hMSCs by acting on cell-cyclic genes including *c-Myc* and *cyclin D1*[45,46]. These diverse effects on cells were also shown in proliferating hDSCs including DPSCs and PDLSCs.

Meanwhile, Sirtuins (also called SIRT protein) has been known to be an important modulator for the survivor of cancer cells; inhibitors of Sirtuins were reported to induce cell death of the human breast carcinoma cell lines[47]. Resveratrol has been also reported to inhibit proliferation and to promote apoptosis of neuroblastoma cells by activate Sirt-1[48]. In addition there are numerous evidences showing that quecertin and curcumin have suppressive effect on the proliferation of cancer cells[49-52]. In our study suppressive effect of quercetin and curcumin on proliferation of cells were also related with the previous studies on various cell types including human and rat adipose stromal cells and osteoblastic cell lines[45,53-54]. Circumin also have been reported to affect apoptosis by suppressing AP-1[55] but this mechanism is still unclear.

The effects of resveratrol, quercetin and curcumin on mineralization of hDSCs (human dental stem cells)

The results of Alizarin red S staining on hDSCs after 2 and 3 weeks of differentiation were shown in Figure 9 to 14. The mineralization of DPSCs treated by resveratrol increased at 1 μM and 10 μM concentrations on 14 days (Fig. 9A). DPSCs treated by quercetin showed increased mineralization after 2 weeks of differentiation with dose dependent manners (Fig. 10A). And DPSCs treated by quercetin showed fastest mineralization and showed most mineralization nodules after 2 weeks of differentiation (Fig. 10). DPSCs treated by curcumin showed most increased mineralization at 1 μM concentration of curcumin after 2 weeks of differentiation (Fig. 11A). DPSCs treated by curcumin showed slowest mineralization and showed least mineralization nodules compared to DPSCs treated by resveratrol or quercetin after 2 weeks (Fig. 9, 10, 11).

The mineralization of PDLSCs treated by resveratrol increased in 1 μM and 5 μM concentrations at 14 days (Fig. 9A). PDLSCs treated by quercetin showed increased mineralization after 2 weeks of differentiation at 5 μM and 10 μM of quercetin (Fig. 10A). But they showed less numbers of mineralization nodules compared to PDLSCs treated by resveratrol or quercetin after 2 weeks of differentiation (Fig. 9B, 10B, 11B). The mineralization of DPSCs treated by resveratrol for 3 weeks increased with dose

dependent manners (Fig. 12A). DPSCs treated by quercetin showed increased mineralization after 3 weeks of differentiation at 1 μM and 5 μM of quercetin (Fig. 13A). DPSCs treated by quercetin showed most numbers and biggest sizes of mineralization nodules compared to DPSCs treated by resveratrol or curcumin after 2 weeks (Fig. 13B). These results were consistent with the results of 2 weeks of differentiation. DPSCs treated by curcumin showed most increased mineralization at 1 μM and 5 μM concentration of curcumin after 3 weeks of differentiation (Fig. 14A). But DPSCs treated by curcumin showed slowest mineralization and least mineralization nodules compared to DPSCs treated by resveratrol and quercetin after 3 weeks (Fig. 12, 13, 14). And these results were related with the results of 2 weeks of differentiation.

The mineralization of PDLSCs treated by resveratrol for 3 weeks increased with dose dependent manners especially at 10 μM concentration of resveratrol (Fig. 12A). And the differences in the numbers or sizes of mineralization nodules seemed to be more significant among the three materials. PDLSCs treated by resveratrol showed most differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to the PDLSCs treated by quercetin or curcumin after 3 weeks of differentiation. PDLSCs treated by quercetin showed mostly increased mineralization after 3 weeks of differentiation at 5 μM and 10 μM concentrations of quercetin (Fig. 14A) compared

to the control group. PDLSCs treated by quercetin showed more differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to the PDLSCs treated by curcumin, but showed less differences compared to PDLSCs treated by resveratrol after 3 weeks of differentiation. But PDLSCs treated by curcumin showed less differences in the numbers or sizes of mineralization nodules between control and experimental groups compared to PDLSCs treated by resveratrol or quercetin after 3 weeks of differentiation.

The results of Real-time PCR (Polymerase chain reaction) were shown in Figure 15 and 16 (n=3). All of the data, C_t-values were analyzed by $\Delta\Delta C_t$-value methods. In the case of the DPSCs treated by 10 μM concentration of resveratrol after 3 weeks of differentiation were shown in Figure 15A. Expression of Col I and OCN was slightly increased. But expression of Runxs2 and OPN showed two-fold and three-fold increase respectively(Fig. 15A). The results of real-time PCR for the PDLSCs treated by 10 μM concentration of resveratrol after 2 weeks of differentiation were shown in Figure 15B. Expression of Col I and Runx2 was slightly increased, expression of OPN was slightly decreased (Fig. 15B). The results of real-time PCR for the DPSCs treated by 1 μM concentration of quercetin after 3 weeks of differentiation were shown in Figure 16A. Expression of Col I and OCN was slightly increased, expression of Runx2 three-fold increased (Fig. 16A). The results of real-time PCR for the

PDLSCs treated by 1 μM concentration of quercetin after 2 weeks of differentiation were shown in Figure 16B. Expression of Runx2 was slightly increased but expression of Col I was slightly decreased. Expression of OCN showed three-fold increase (Fig. 16B).

It has been known that Wnt-signaling pathway is strongly associated with osteogenesis[56], but simultaneously, it has been known to inhibit the osteogenesis of mesenchymal stem cells when the degree of active Wnt-signalling is low[57]. There are accumulating evidences showing that Wnt regulates Runx2 expression, but canonical Wnt down regulates osteogenesis in human MSCs[58]. Therefore, three compounds examined in this study may partially act on the differentiation of hDSCs by modulating those series of Wnt-related mechanisms mentioned above.

According to the several studies the rate of osteogenesis decreased under highly active PPARγ while the rate of adipogenesis increased[59].

Considering the fact that the chemical structures of the three compounds are similar to phytoesterogens, which mainly target estrogen receptors (ER receptors), effects of the three compounds on hDSCs' differentiation can also be explained by the ER receptor-induced activation of PPARs[59]. Phytoestrogens are known as weak estrogens which can be found in plants. Or the plants-derived materials which can be converted to bio-effective forms by human metabolism[60]. They also have been considered

as effective bio-materials for cancers including breast cancers, prostate cancers and colon cancers[61,62]. Mainly, it has been hypothesized to affect bone formation and gained interests of the effective bio-materials for osteoporosis[63]. Thus, resveratrol, qeurcetin and curcumin might be said to have properties of promoting bone formation, and suppressing cancer cell proliferation simultaneously.

It had not been determined the exact mechanisms for up-regulating osteogenic markers by resveratrol, quercetin and curcumin yet. But there are many evidences that they can affect bone healing process by up-regulating the osteomarkers including OPN, Runx2, BMP-2 or BMP-7[64]. There are lack of reports about the up-regulation of osteogenic markers on hDSCs by resveratrol, quercetin and curcumin, but they are hypothesized to promote expressions of OPN, Runx2 and Col I in several cell types including human PDL fibroblasts[65], human gingival fibroblasts[66]. The candidating pathways for the up-regulation of osteogenic markers are known as Wnt-signaling, Sirt-1. Thus the results of real-tim PCR in this study were consistent with the previous researches for mineralization on human dental tissues. Still, the further studies are needed for the optimal doses of natural phenols on mineralization, and the mechanisms on the up-regulations of mineralization markers.

The natural phenols, resveratrol, quercetin and curcumin might be one of the safe and effective biomaterials for regenerative dentistry

These three plant-derived compounds stimulated the proliferation under low concentrations while inhibiting it under high concentrations. This results were consistent with the facts that theses materials have been reported as a suppressors for the cancer cells and promotors for the proliferation of human stem cells including hDSCs and hMSCs. So the basic idea for this research was that these compounds have similar chemical structures, and have similar cellular effects by acting on the overlapping molecular targets based on the bio-informatics. Even though specific molecular targets for these materials were not defined in this research, we showed that they promoted proliferation under low concentrations, especially 1 μM, and they induced apoptosis under high concentrations over 50 μM. This indicates that these natural phenols can be used both relating with the regenerative medicine and the cancer therapies.

They also promoted mineralization of DPSCs and PDLSCs 2 or 3 weeks after the treatment of three compounds. They showed promoted mineralization under the range of concentrations 1 μM to 10 μM, especially 5 μM. This results indicate that these natural phenols have positive effects on mineralization of hDSCs slightly overdose for the maximum increasing dose for the pro-

liferation, 1 μM.

Thus resveratrol and quercetin could be considered as promoting proliferation and mineralization with low concentrations of 1 μM to 10 μM on hDSCs, at least PDLSCs and DPSCs. And with these concentrations, there might be no cytotoxic effects on the hDSCs. Curcumin has the least promoting effect on proliferation of hDSCs, but it showed promoted mineralization with low concentration 1 μM to 10 μM also.

With high concentrations over 50 μM, resveratrol, quercetin and curcumin showed decreased proliferation on hDSCs, escpecially on the PDLSCs. Cell deaths were shown and these might be related with apoptosis. The relationship between the concentrations of these compounds and the cell deaths remains for the further studies. And the further studies on mechanisms on the cell deaths are needed.

V. Conclusion

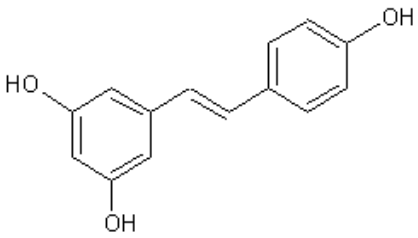
Resveratrol, quercetin and curcumin have been reported that they promoted proliferation on hMSCs, and suppressed proliferation on cancer cells. The aim of this study was to investigate on this controversial topic on the natural polyphenols through bio-informatics based on the chemical structures.

In summary, our results indicate that the safe and effective range of concentrations for regenerative dentistry might be 1 μM to 10 μM. Resveratrol showed most promoted proliferation on hDSCs at 1 μM concentration, quercetin and resveratrol showed most promoted mineralization of DPSCs and PDLSCs at 5 μM concentration respectively. And these three compounds inhibited proliferation of hDSCs induced by which thought to be an apoptosis under the concentrations over 50 μM, especially with quercetin and curcumin. These results were also consistent with the reports that resveratrol, quercetin and curcumin could be the related bio-materials for the cancer research. And they could be the useful natural compounds related for the regeneration using dental stem cells (Fig. 17).

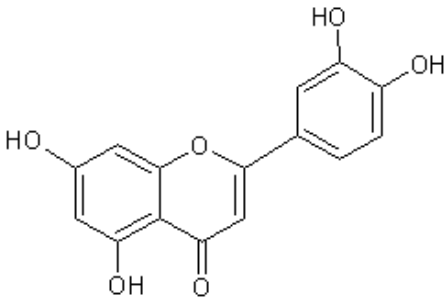
Table 1. Primers for real-time PCR

Gene	Accession No.	Sequences
HPRT	NM_000194	Forward: 5'-GCTATAAATTCTTTGCTGACCTGCTG-3' Reverse: 5'-AATTACTTTTATGTCCCCTGTTGACTGG-3'
Col I	NM_007742	Forward: 5'-TCTCCATCCTTGCCGTTGATTG-3' Reverse: 5'-TCCCCACCTTCAAAATTCTGGG-3'
OPN	X16575	Forward: 5'-CATTGCAGGTCTCCTGGAACAA-3' Reverse: 5'-TTAGCATCGGTGGTTTCCGTTC-3'
OCN	X53698	Forward: 5'-GTGCAGAGTCCAGCAAAGGT-3' Reverse: 5'-TCAGCCAACTCGTCACAGTC-3'
RUNX2	NM_001015051	Forward: 5'-TGGAACATCTCCATCAAGGCAG-3' Reverse: 5'-TCAGGATATTCGGGACGTTGGA-3'

(A)



(B)



(C)

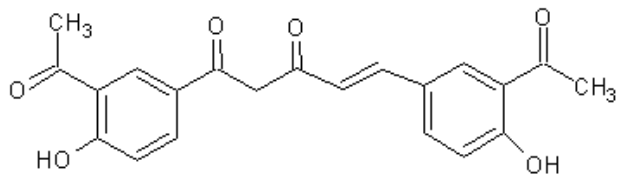
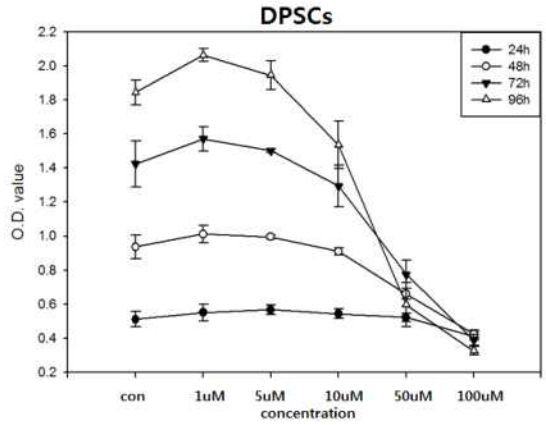


Figure 1. Two dimensional structures of (A) resveratrol, (B) Quercetin and (C) Curcumin.

(A)



(B)

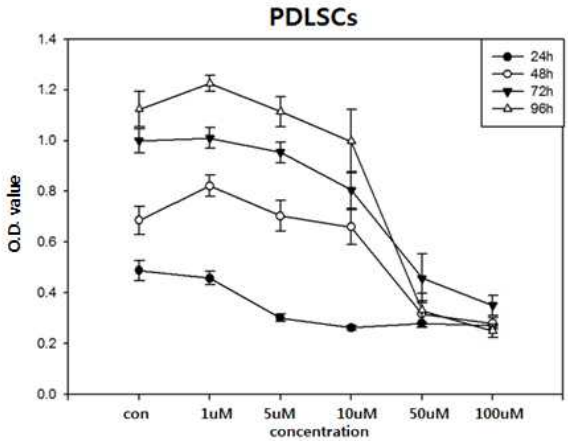
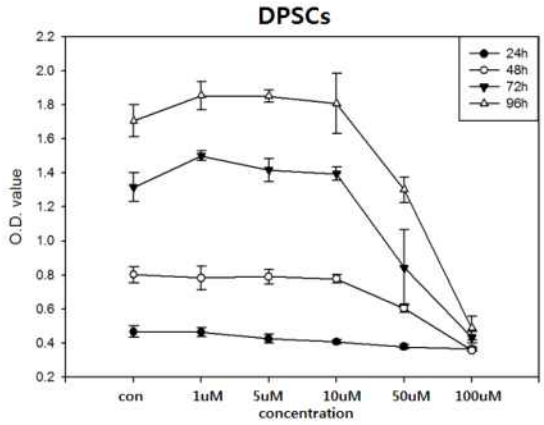


Figure 2. The effect of various concentrations of Resveratrol on the proliferation of hDSCs.

(A)



(B)

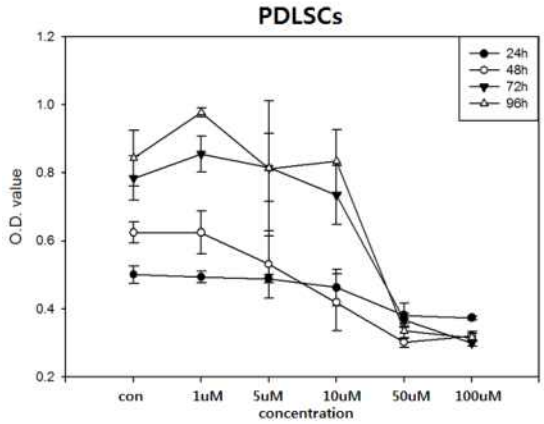
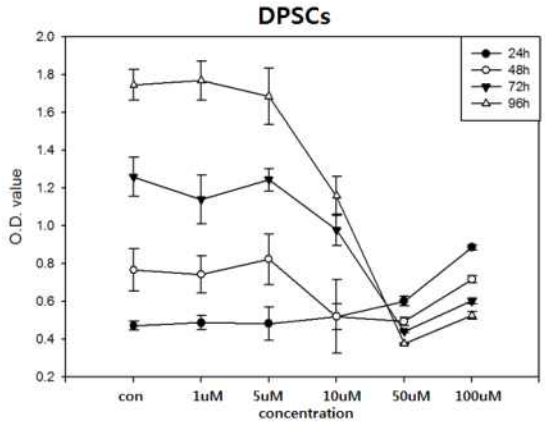


Figure 3. The effect of various concentrations of Quercetin on the proliferation of hDSCs.

(A)



(B)

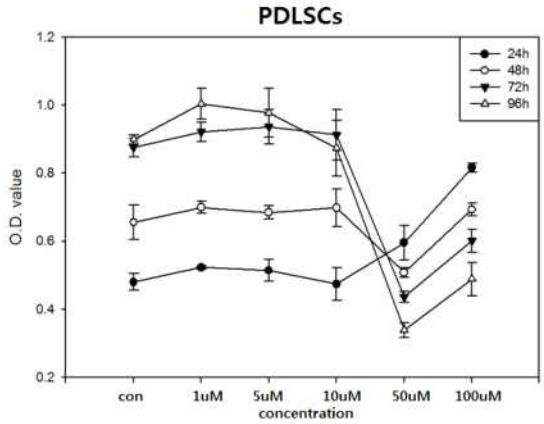
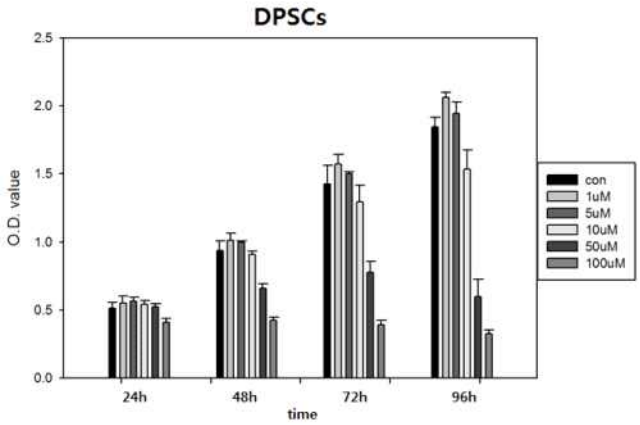


Figure 4. The effect of various concentrations of Curcumin on the proliferation of hDSCs.

(A)



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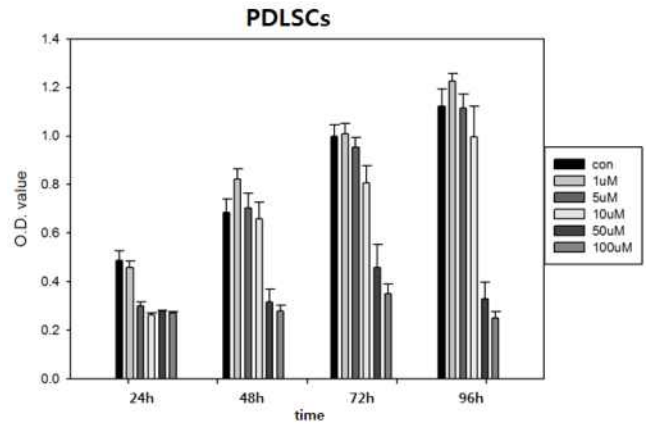
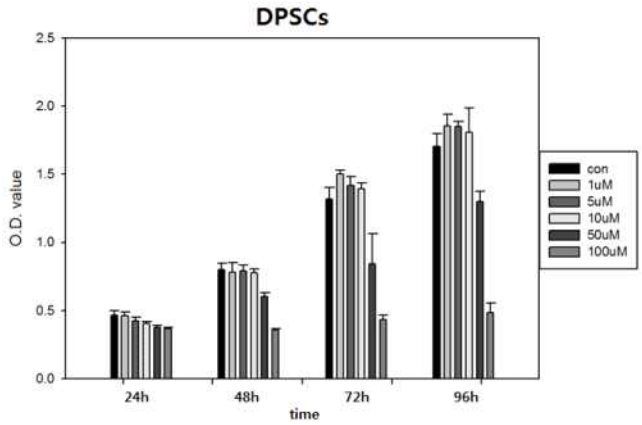


Figure 5. The effect of various concentration of resveratrol on the proliferation of hDSCs.

(A)



(B)

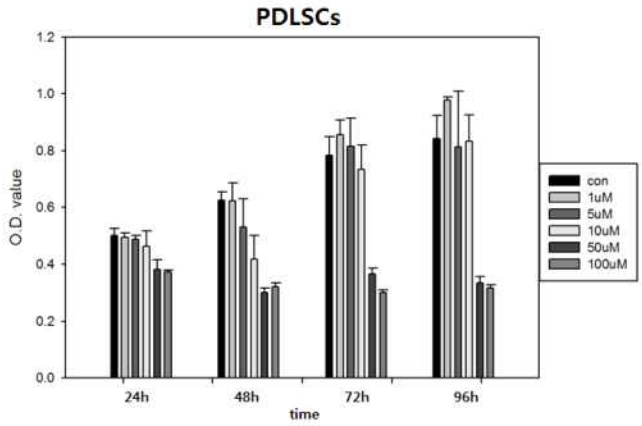
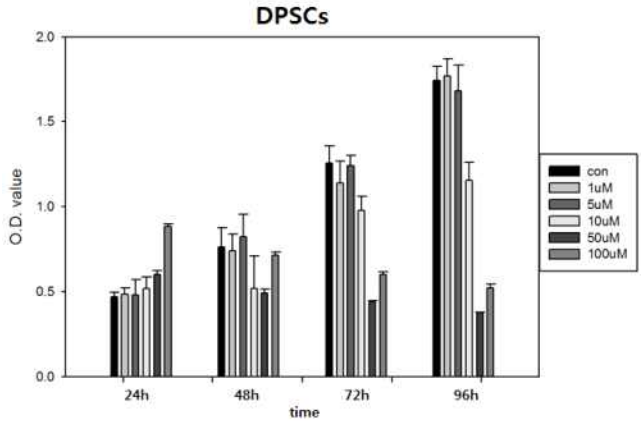


Figure 6. The effect of various concentration of quercetin on the proliferation of hDSCs.

(A)



(B)

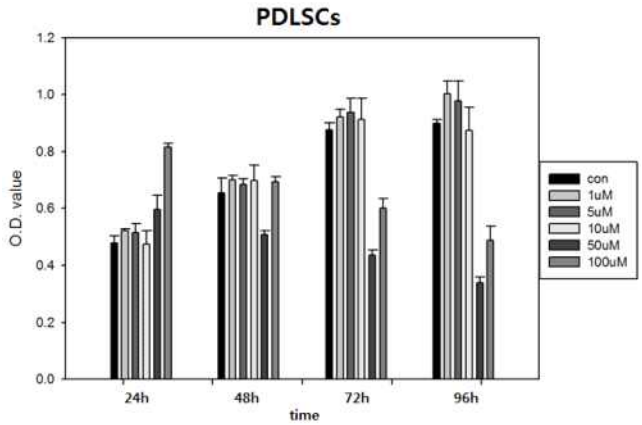


Figure 7. The effect of various concentration of curcumin on the proliferation of hDSCs.

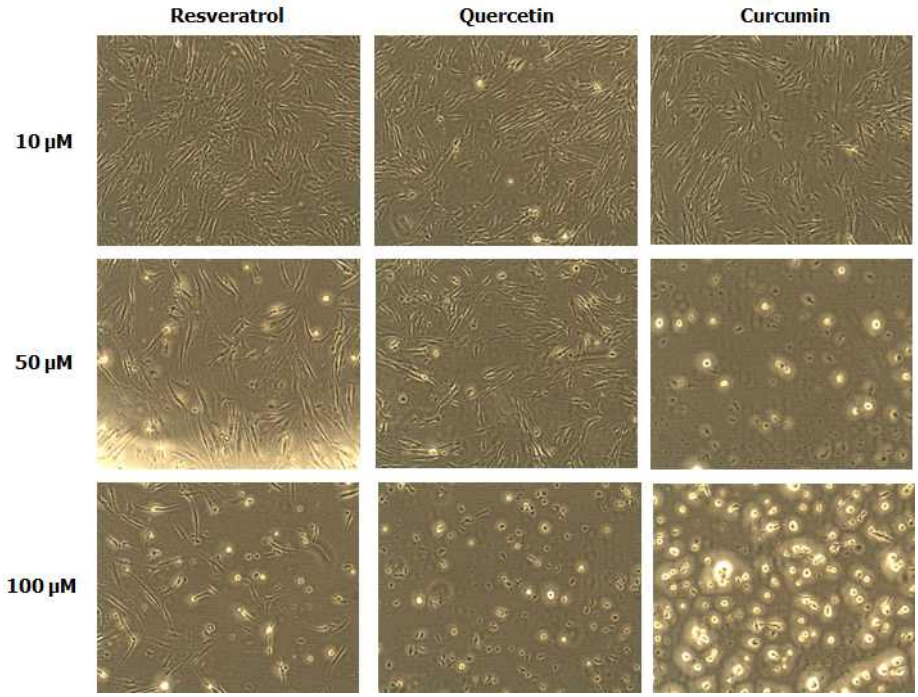
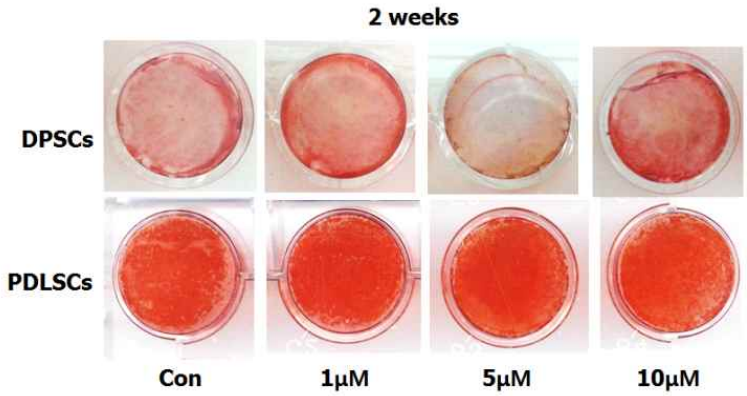


Figure 8. The cytotoxic effects at high concentrations of resveratrol, quercetin and curcumin on the proliferation of hDSCs after 48h.

(A)



(B)

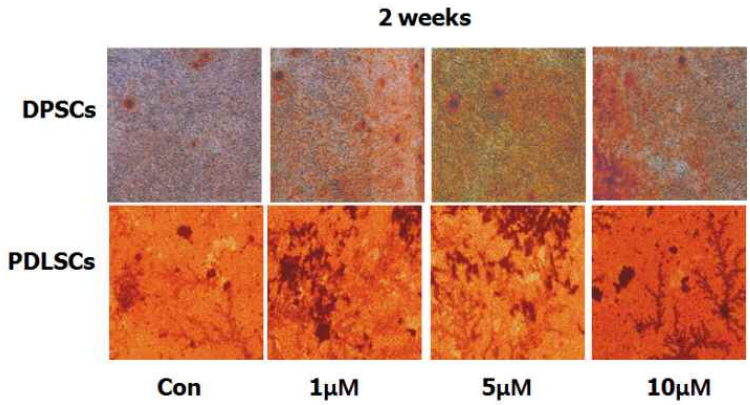
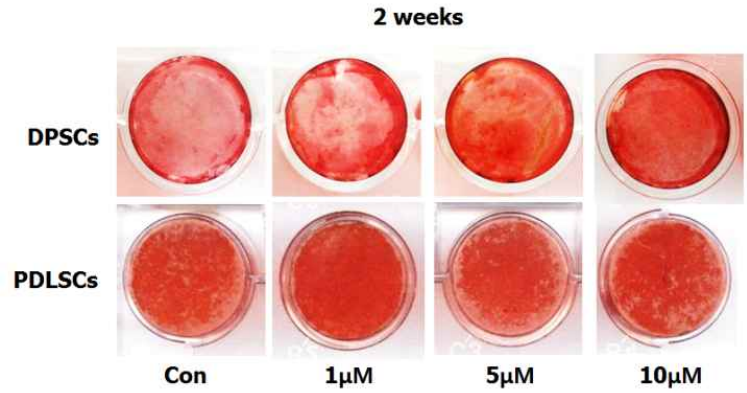


Figure 9. The effects of resveratrol on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by resveratrol for 14 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

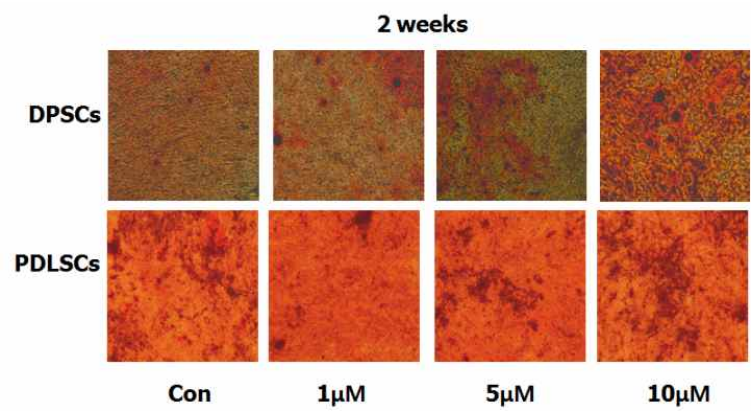
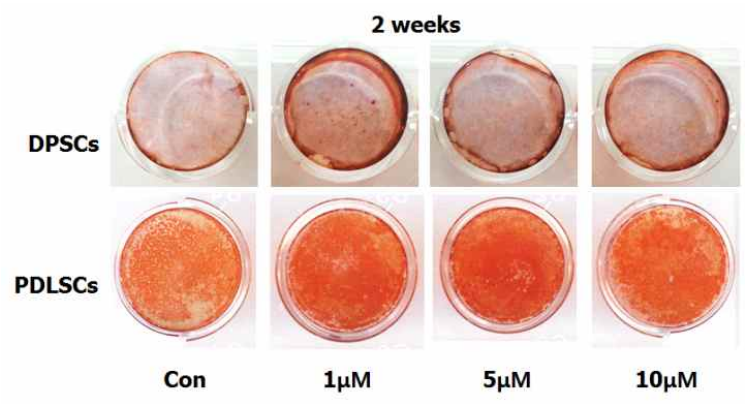


Figure 10. The effect of quercetin on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by quercetin for 14 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

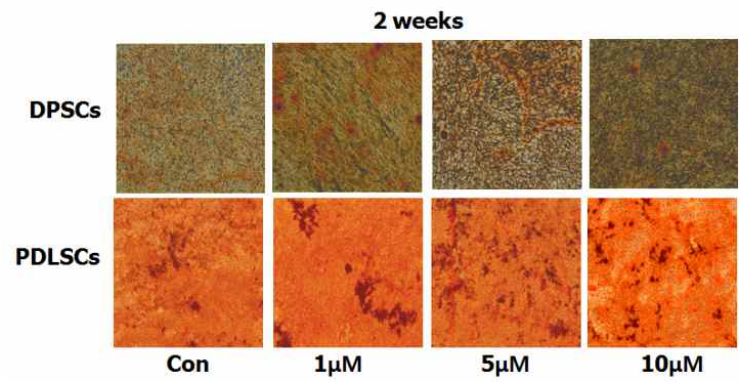
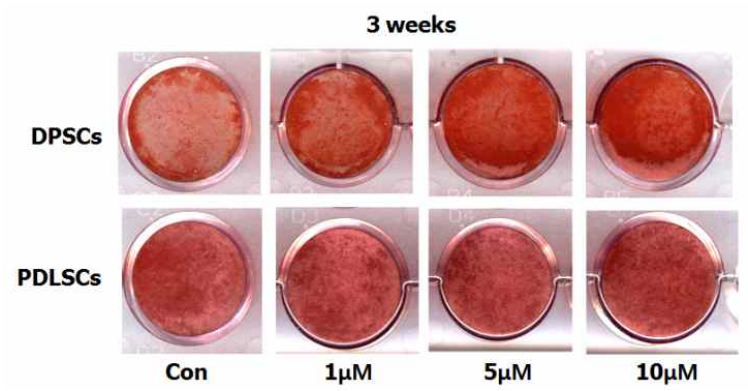


Figure 11. The effect of curcumin on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by curcumin for 14 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

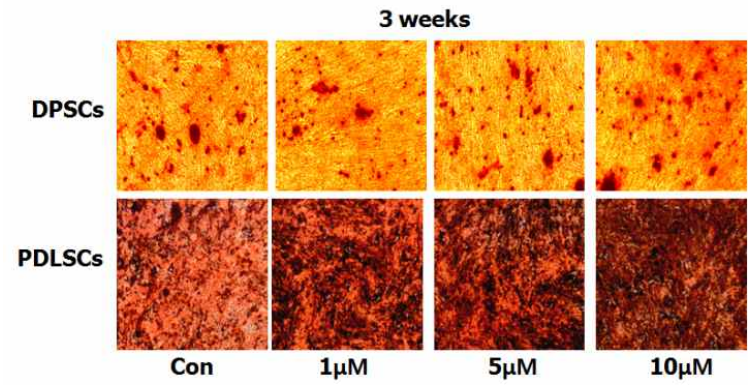
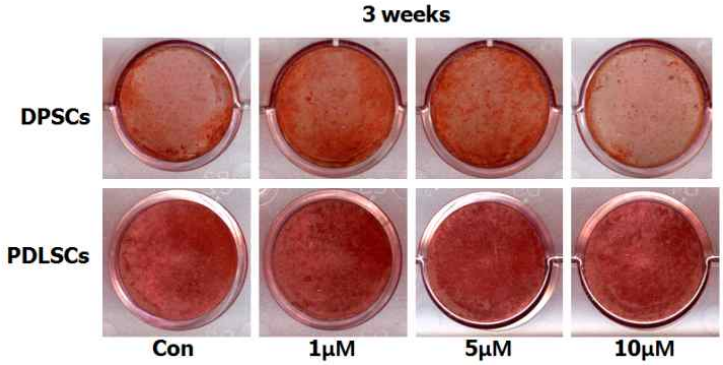


Figure 12. The effect of resveratrol on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by resveratrol for 21 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

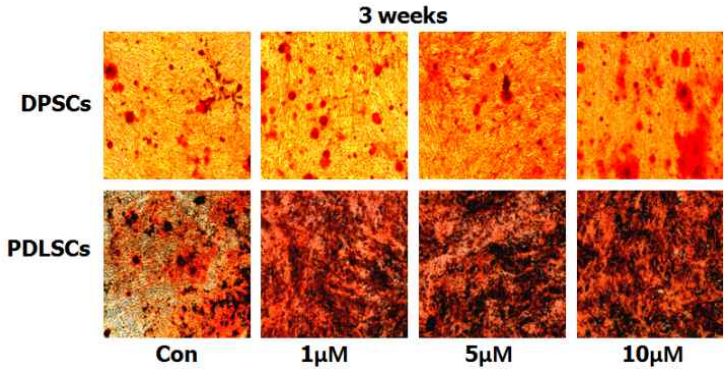
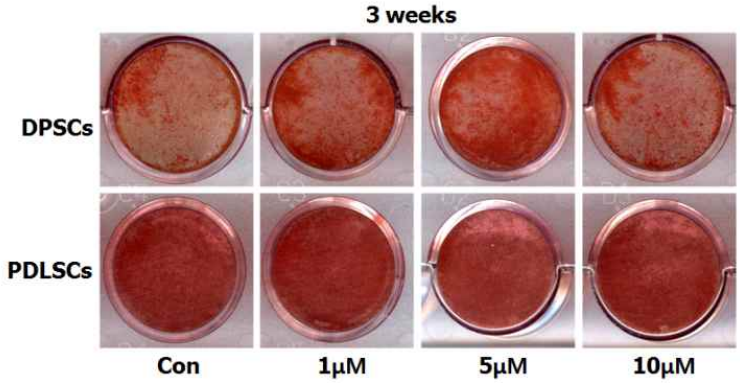


Figure 13. The effect of quercetin on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by quercetin for 21 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

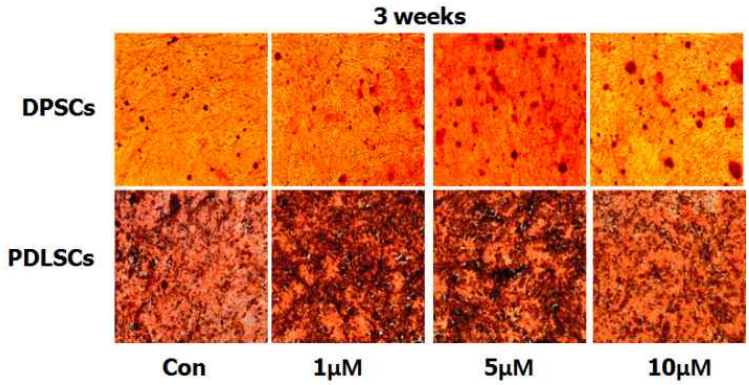
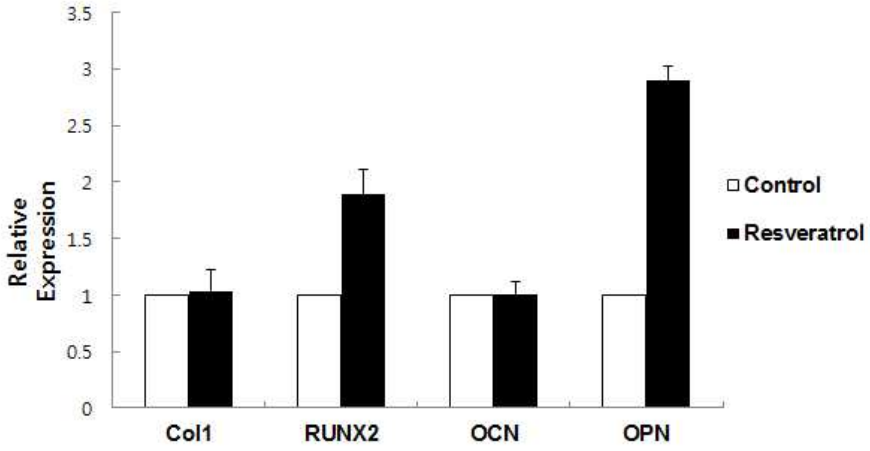


Figure 14. The effect of curcumin on the mineralization of hDSCs (Alizarin red S staining)

The mineralization level of hDSCs treated by curcumin for 21 days evaluated by Alizarin red S staining (A), and there were Alizarin red S-positive nodules shown in (B).

(A)



(B)

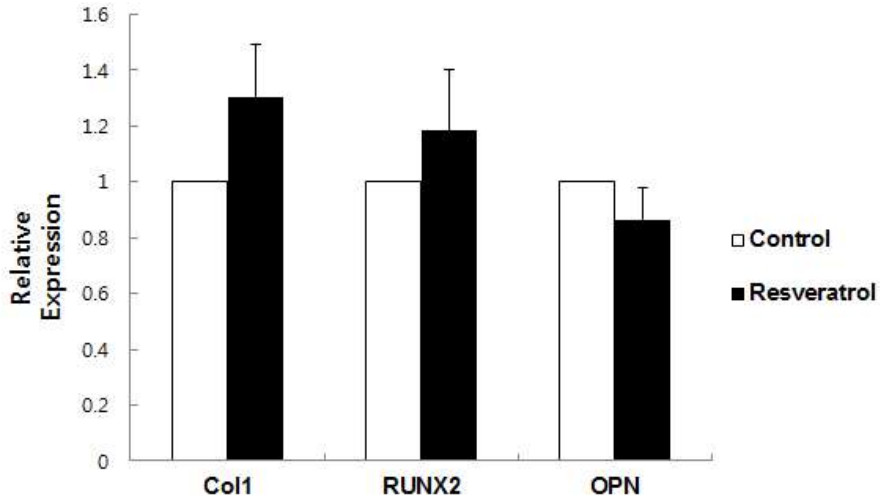
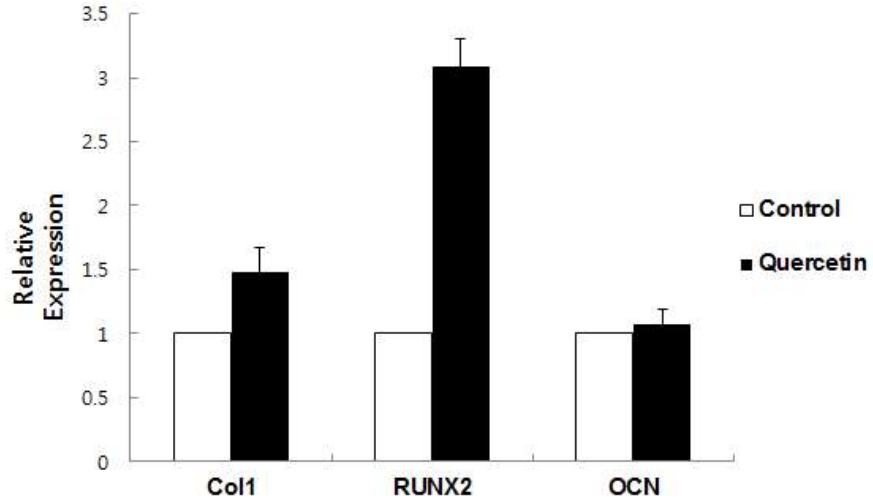


Figure 15. Real-time PCR (Polymerase chain reaction) with several primer sets on (A) 3 weeks of DPSCs and (B) 2 weeks of PDLSCs treated by resveratrol.

(A)



(B)

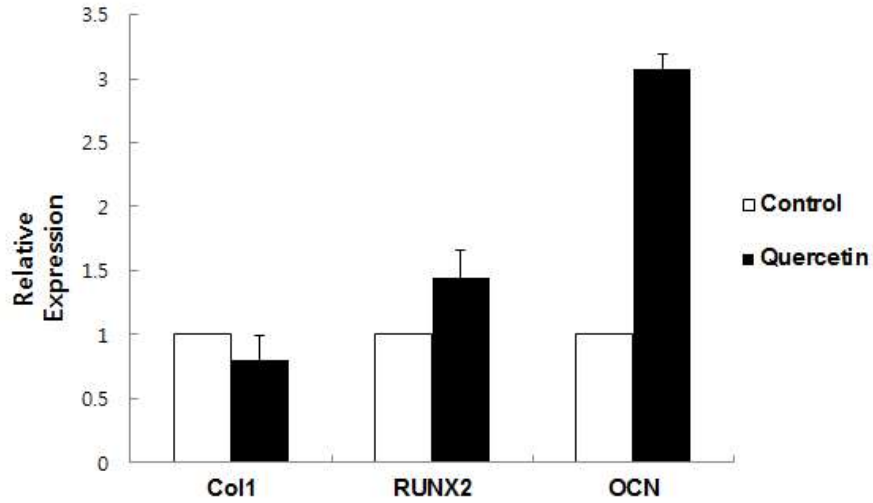


Figure 16. Real-time PCR (Polymerase chain reaction) with several primer sets on (A) 3 weeks of DPSCs and (B) 2 weeks of PDLSCs treated by quercetin

	Resveratrol				
Concentration	1 μM	5 μM	10 μM	50 μM	100 μM
Proliferation DPSCs	++	+	=	-	- -
Proliferation PDLSCs	+	=	-	- -	- -
Mineralization of DPSCs on 14 days	+	=	++	/	/
Mineralization of DPSCs on 21 days	+	+	++	/	/
Mineralization of PDLSCs on 14 days	+	++	+	/	/
Mineralization of PDLSCs on 21 days	++	+++	+++	/	/

	Quercetin				
Concentration	1 μM	5 μM	10 μM	50 μM	100 μM
Proliferation DPSCs	+	+	+	-	- -
Proliferation PDLSCs	+	=	-	- -	- - -
Mineralization of DPSCs on 14 days	+	+++	++	/	/
Mineralization of DPSCs on 21 days	+	+++	++	/	/
Mineralization of PDLSCs on 14 days	=	+	++	/	/
Mineralization of PDLSCs on 21 days	++	+++	+++	/	/

	Curcumin				
Concentration	1 μM	5 μM	10 μM	50 μM	100 μM
Proliferation DPSCs	=	=	=	- -	- - -
Proliferation PDLSCs	+	+	=	- - -	-
Mineralization of DPSCs on 14 days	=	+	=	/	/
Mineralization of DPSCs on 21 days	+	++	++	/	/
Mineralization of PDLSCs on 14 days	+	++	+	/	/
Mineralization of PDLSCs on 21 days	++	+++	+	/	/

Means the most effective dose

Means the experimental group showed cell death mostly

Figure 17. The effect of resveratrol, quercetin and curcumin on the proliferation and mineralization of hDSCs. This diagram is not drawn in scale. Notations of (+), (=), (-), mean promotion, on a par, suppression of proliferation or mineralization compared with the control group respectively.

VI. References

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국문초록

식물 유래 화합물인 Resveratrol, Quercetin, Curcumin이 사람 치아 줄기세포에 미치는 영향

지도교수 정 필 훈

김 민 수

서울대학교 치의학대학원 치의학과

식물로부터 유래한 화합물인 레스베라트롤, 퀘세틴, 커큐민의 생물학적 효과에 대한 연구들이 많이 이루어져왔으나 사람 치아 줄기세포에 대한 효과에 대한 연구는 거의 이루어진 바가 없다. 이 세 종류의 화합물은 화학적으로 유사한 구조를 가지고 있으며, 특히 대장암 치료를 비롯하여 각종 만성 질환에 있어서도 세 물질이 유사한 긍정적 효과를 지니는 것으로 보고된 바 있다. 특히 사람 중배엽 줄기세포의 증식을 증가시키며, 암세포의 증식을 억제하는 효과를 공통적으로 가짐이 보고되어 왔다. 이러한 사실은 위의 세 물질이 세포에 가지는 효과가 다소 상반됨을 나타내며, 동시에 위 세 물질이 더 넓은 범위에서 유용하게 사용될 수 있음을 시사하기도 한다.

사람 치아 줄기세포는 재생 치의학 분야에서 그 중요성이 강조되어 왔고 어떤 물질에 의해 어떻게 분화되어 어떤 재생에 쓰이는지는 연구 중이다. 따라서 많은 후보 물질들이 제기되어 왔음에도 재생과 관련하여서는 연구 초기 단계이다.

따라서 본 연구의 목적은 화학적 구조를 기반으로 한 생물 정보학적 접근을 통하여 폴리페놀이 안전하면서도 효과적인 재생 치의학의 후보 물질이 될 수 있음을 보이고자 하는 것이다. 적어도 치수 줄기세포와 치주인대 줄기세포를 포함하는 사람 치아 줄기세포에 있어 증식과 분화에 미치는 영향을 살펴보고자 하였다. 또한 사람 치아 줄기세포에 미치는 영향과 레스베라트롤, 퀘세틴, 커큐민의 농도간에 어떠한 관계가 있는지를 밝히고자 하였다.

사람 치아 줄기세포의 증식은 MTT 기법을 통해 24 시간, 48 시간, 72 시간, 96 시간에 걸쳐 측정하였으며 사람 치아 줄기세포의 광화는 2주, 3주에 걸쳐 Alizarin red S 염색법을 통해 관찰하였다. 또한 중합효소연쇄반응을 통해 유전자의 발현을 확인하였다.

레스베라트롤, 퀘세틴, 커큐민을 처리한 실험군에서 대체로 낮은 농도에서 사람치아줄기세포 증식이 대조군에 비해 증가하였으며 고농도에서 증식이 억제되거나 세포 독성이 있음을 확인하였다. 또한 Alizarin 염색법을 통해 세 가지 물질을 처리한 실험군에서 사람치아줄기세포 광화가 촉진됨을 확인하였고 중합효소연쇄반응법을 통해 이를 재확인하였다.

우리의 결과는 1 μM 와 10 μM 농도 사이에서 위 물질들이 안전하고 효과적인 재생 치의학의 후보물질이 될 수 있음을 시사한다. 레스베라트롤은 1 μM의 저농도에서 사람 치아 줄기세포의 증식을 가장 크게 증가시키는 효과를 보였고, 5 μM 농도에서 치주인대 줄

기세포의 분화를 촉진시켰다. 퀘세틴 역시 5 μM 농도에서 치수 줄기세포의 분화를 촉진시켰다. 세 물질 모두 50 μM이 넘는 고농도에서는 세포사멸로 추측되는 기전을 통해 세포 수의 감소를 보였으며 이는 퀘세틴과 커큐민에서 특히 뚜렷하게 관찰되었다. 이런 결과들은 레스베라트롤, 퀘세틴, 커큐민이 암 연구와 관련이 있다는 일련의 결과들과 일맥상통 한다. 그리고 천연 화합물인 위 세 물질이 치아 줄기 세포의 재생 연구에도 유용하게 쓰일 수 있음을 확인하였다고 할 수 있다.

주요어: 사람 치아 줄기세포, 레스베라트롤, 퀘세틴, 커큐민, 증식, 분화

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