The possibility of genistein as a new direct pulp capping agent

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Genistein, kind of soy isoflavones, is well-known as natural ingredients and consumed as health foods and supplements. They are expected to improve renal function. They have high-affinity to estrogen receptor \( \beta \) expressed predominantly in bone tissue, they prevent osteoporosis specifically and safely. We examined whether genistein can be a new direct capping agent. In this study, we examined the effect of genistein for the proliferation and differentiation of rat dental pulp cells \textit{in vitro} and the ability of tertiary dentin formation \textit{in vivo}. As a result, rat dental pulp cells with genistein were increased activity of ALPase and showed alizarin red positive-staining. Calcification-related genes expression has been confirmed by the addition of genistein. From \textit{in vivo} study, high quality of tertiary dentin formation and minor pulp reaction were observed. From these findings, it was suggested that genistein may be useful agent for direct pulp capping.

\textbf{Keywords:} Genistein, Direct pulp capping, Rat dental pulp cells

INTRODUCTION

Soy isoflavone, which are natural ingredients, are classified flavonoid and it has been applied for food or supplement for in our life. Soy isoflavone attracted attention from biomedical field because of their effects that such as anti-oxidant effect, suppression of breast cancer or improvement of renal function\textsuperscript{1-3}. Soy isoflavone is metabolic products formed during the growth of soybean and their content is approximately 1.4 mg per 1 g of soybean\textsuperscript{4}. And also soy isoflavone have been classified to genistein, daidzein and glycitein. Although these soy isoflavones does not have a steroid skeleton, this molecular structure is similar to that of estrogen\textsuperscript{5}. They indicated the specific affinity to the estrogen receptor which is expressed in bone tissue predominantly\textsuperscript{4-7}. It has been reported that genistein has stronger estrogen-like effect than the other flavonoids\textsuperscript{8}, induce the activation of osteoblasts and increase the bone mass, and said to be effective in improvement and prevention of osteoporosis\textsuperscript{8,9}. Soy isoflavones are also highly safe\textsuperscript{10,11} because it is a natural ingredient derived from food, so side effects such as anti-oxidant effect, suppression of breast cancer and prevention of osteoporosis biphosphonate drug therapy has not been reported.

Accidental pulp exposure is not in a low frequency for our daily clinical treatment. In this case, calcium hydroxide preparations have been traditionally used for direct pulp capping agent. Calcium hydroxide is widely used and they are effective, inexpensive and safe to induce formation of tertiary dentin. However, calcium hydroxide has highly alkaline (pH12), which can be formed below of pulp necrosis following cause chronic inflammation and formed internal absorption layer. To make matters worse, the tertiary dentin which is formed so as to cover the surface of exposed pulp as a protective response have such as defects and tunnel-like shaped, their presence will be caused for deterioration of pulp disease\textsuperscript{12-15}. Therefore, development of highly secure direct pulp capping agent to induce the high quality tertiary dentin more effectively has been demanded.

Currently many cytokines or bonding agents are enabled to calcification of the dental pulp, and clinical application has been operated as direct pulp capping agents\textsuperscript{16-18}. But few calcification inducing substance for safety and derived from natural ingredients has been reported. Recently, Mineral Trioxide Aggregate (MTA) is excellent pulp capping or pulpotomy\textsuperscript{19,20} agents because of their high biocompatibility. Not only MTA, Er:YAG leaser is also useful tools for direct pulp capping equipment\textsuperscript{21}. In this experiment, we focused on the effect of soy isoflavones promote osteoblast differentiation, we investigated whether genistein affect differentiation and calcification of rat dental pulp cells.

The authors studied that whether genistein induced proliferation and differentiation for rat dental pulp cells \textit{in vitro} and affected tertiary dentin formation and pulp inflammation \textit{in vivo}.

MATERIALS AND METHODS

\textbf{Tissue culture}

Dental pulp tissues were obtained from 12 weeks-old male Wister rat (Hokudo, Sapporo, Japan) lower incisor, according to a procedure of Handa et al.\textsuperscript{22}. Their tissues were digested sequentially 4 times with 3 mg/mL of bacterial collagenase (Roche Diagnostics, Basel, Switzerland) in phosphate-buffered saline. The first populations occupied with the unattached blood cells (data not shown) were discarded and the last three were incubated in 35 mm dishes (BD-Falcon Biosciences, Lexington, TN, USA) with Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, Irvine, UK) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA).
in a humidified atmosphere of 5% CO₂ at 37°C. When the cells reached approximately 80% confluence, they were passaged with TrypLE Express (Invitrogen) and maintained as Rat dental pulp cells (RDP). We harvested into 60 mm dish (BD Falcon Biosciences) and the medium was changed every 2 days. Experimental procedures involving the use of animals were reviewed and approved by the animal experiment committee of Health Sciences University of Hokkaido.

**Measurement of cell proliferation**

1) RDP was plated into 96-well plate (BD-Falcon Biosciences) at a density of 2×10³ cells per well, and incubated for 48 h. Then, we incubated the RDP in the presence of genistein (Wako, Tokyo, Japan) with various concentrations 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM (G0.01, G0.1, G1, G10, G100 respectively) and without genistein as a control. By using Alamar blue (Biosource International, Camarillo, CA, USA), we measured proliferation rate of RDP, according to the manufacturer’s instructions. The absorption spectrometer (iMark™, BIO-RAD, Hercules, CA, USA) was used for measuring fluorescence in 96-well plates at wavelengths of 570 nm and 595 nm emission.

2) We measured tyrosine kinase (TK) activity by using Universal Tyrosine Kinase Assay kit (TaKaRa, Shiga, Japan), with Enzyme-Linked Immuno Sorbent Assay (ELISA) methods following the manufacturer’s protocol. RDP were plated into 35 mm dish (BD-Falcon Biosciences) at a density of 5×10⁴ cells and incubated for 24 h. The samples were scrapped after 48 h incubated with genistein. The values were evaluated as rate of TK activity per amount of total protein contained in the cells. The amount of total protein was calculated by the absorbance with BCA protein assay kit (PIERCE, Rockford, IL, USA). These experiments carried out three times repeated independently.

3) We seeded RDP at a concentration of 1×10⁴ cells/dish to the 60 mm dish. After 24 h for cell seeding, we harvested under various concentrations of genistein for 1 h. Finally, in order to promote cell proliferation, RDP were incubated with 10 ng/mL of Platelet-Derived Growth Factor-AA (PDGF-AA, Pepro Tech, Rocky Hill, NJ, USA) for 10 min. After incubation, cells were collected and Akt phosphorylation activity was measured in the supernatant by using Human/Mouse/Rat Phospho-Akt (S473) Pan Specific Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol.

**Cell differentiation and calcification in the presence of genistein**

We harvested RDP into 24-well plate (BD-Falcon Biosciences) at a density of 4×10⁵ cells per well and incubated for 48 h. And then, we added various concentrations of genistein to RDP.

1) To evaluate the ALPase activity, we collected the samples by mechanically peeling on 10, 17 and 30 days period using 0.1% Triton X (Sigma-Aldrich) in H₂O. After centrifugate, supernatant of the samples were measured with a Lab Assay ALP kit (Wako), according to the manufacturer’s protocol. We evaluated the values as the score of ALPase activity per amount of total protein contained in the cells. Evaluations of ALPase activity were analyzed after the calculated average and standard deviation each groups. This experiment carried out independently triplicate.

2) To see the calcified nodule formation in RDP, incubated for 30 days with genistein, fixed with 10% formalin solution and stained with 2% Alizarin-red S (Wako) solution for 20 min. Then, we observed the nodule formation with a microscope.

3) We examined the mRNA expressions for marker-related molecules of odontoblast such as Runt-related transcription factor 2 (Runx2), Dentin sialophosphoprotein (DSP), Dentin matrix protein-1 (DMP-1), Alkaline phosphatase (ALPase), Bone sialoprotein (BSP), Osteocalcin (OCN), Osteopontin (OPN), Estrogen receptor α (ERα), β (ERβ) and transforming growth factor-β receptor 1, 2 (TGFβ-R1, 2) were quantified with real time PCR. The levels of OCN protein charged in serum were analyzed using a Rat Gla-Osteocalcin High Sensitive EIA Kit (TaKaRa) by ELISA methods, following the manufacturer’s instructions. We evaluated the value as the levels of OCN per unit amount of protein contained in the cells in the presence of several concentration of genistein for 20 days. We analyzed OCN levels after the calculated average and standard deviation each groups. Next, to quantify the p38 phosphorylation of RDP, cells were cultured for 24 h with genistein, and then we had collected the cells mechanically. The supernatant of the samples were measured for activity of the p38 phosphorylation by using Human/Mouse/Rat Phospho-p38α (T180/Y182) Immunoassay (R&D Systems). Each group was duplicate samples. These experiments were preformed three times independently.

**Direct pulp-capping procedure in vivo**

We used 51 male Wister rats of 8 weeks-old. The rats were anesthetized using an intraperitoneal injection of
We used these sequence-specific primers for PCR.
neutrophils are observed.

Score 1, Severe inflammation (presence of strong vasodilation of blood vessels appearing as an abscess and significant inflammatory infiltration by polymorphonuclear leukocytes and neutrophils is seen throughout the crown).

These areas in the sections were measured by means of Image J (Wayne Rashand, MD, USA).

Statistical analysis
The data were expressed as the mean±standard deviation (SD) and statistically analyzed by Tukey-Kramer tests that calculate the each group (Figs. 1A, 1B, 1C, 2A, 3 and 4) and the Mann-Whitney U test (Fig. 6). The statistical significant level was selected at $p<0.05$.

RESULTS

Measurement of RDP proliferation and TK activity
We observed cell proliferation of RDP affected by addition of various concentration of genistein. The group of low concentration ($G_{0.1}$~$G_{10}$) showed similar cell proliferation to the control while period all period (Fig. 1A). The cell proliferation of the high concentration group ($G_{100}$) was showed similar as well as the other group until 3 days incubation. However, after 5 days, cell proliferation was markedly suppressed and most cells have been stopped proliferation after 10 days (Fig. 1A). Subsequently, we measured of TK activity after incubation for 48 h with various concentration of genistein. The group of low concentration ($G_{0.1}$~$G_{10}$) inhibited of TK activity in a dose-dependent manner up to about 90–65% compared with the control. However, the group of high concentration ($G_{100}$) largely suppressed TK activity up to about 10% compared to the control (Fig. 1B). Moreover, phosphorylation of Akt activity was significant increased after treatment with PDGF-AA alone in the genistein absence group. However, in the presence of genistein, the addition of PDGF-AA did not induce the activation for Akt phosphorylation in a concentration-dependent manner (Fig. 1C).

RDP calcification induced by genistein
To analyze the effects on calcification for RDP by the addition of genistein, ALPase activity was measured and alizarin red staining. ALPase activity of genistein added group showed same value as the control at 10 days incubation. But, after 17 days, genistein added group increased ALPase activity compared with the control (Fig. 2A). Next, alizarin red staining performed at 30 days incubation result in nodule formation $G_{0.1}$, $G_1$ and $G_{10}$. Especially, the nodule formation rate of $G_1$ was significantly higher than the other group. In the control and $G_{0.01}$, nodule formation was not observed (Fig. 2B).

mRNA expression of odontoblast-related marker molecules, ER and TGF-$\beta R$
The real time PCR analysis carried out to evaluate of genistein on the mRNA expression level of odontoblast

![Fig. 1](image-url)  
Cell proliferation by the addition of genistein.
(A): Rate of the cell proliferation RDP was cultured after 48 h, in the presence of genistein and without genistein as a control. The transition of the cell proliferation was stained by using Alamar blue and measured ($n=3$). The asterisks (*) indicate a significant difference ($p<0.05$).

(B): Tyrosine kinase activity
We measured tyrosine kinase activity by using ELISA methods. RDP were collected after 48 h incubated with genistein. Significantly different ($P<0.05$) between the asterisk (*).

(C): Phosphorylation of Akt
Akt phosphorylation was enhanced by the addition of PDGF-AA. Then, we measured the inhibitory effect of Akt phosphorylation by genistein ($n=3$), a–c, indicate significant differences ($P<0.05$) between the same symbols.
Fig. 2  In vitro mineralization assay
(A): ALPase activity
We measured by absorbance of the ALPase activity after 10, 17 and 30 days of cultured with genistein.
(B): Alizarin red staining
RDP were incubated for 30 days with genistein. We observed the nodule formation with a microscope.
Control (a), G0.01 (b), G0.1 (c), G1 (d), G10 (e)

Fig. 3  Analysis by real time PCR
■: Day 10, □: Day 17
After incubation for 10 and 17 days in cultured RDP with genistein, Runx2 (A), DSPP (B), DMP-1 (C), ALPase (D), BSP (E), OCN (F), OPN (G), ERα (H), ERβ (I), TGF-βR1 (J) and TGF-βR2 (K) were quantified by real time PCR.
a–h, indicate significant differences (P<0.05) between the same symbols.
marker molecules, ER and TGF-βR. Those odontoblast markers such as DSPP, DMP-1 and ERβ mRNA expression level of genistein added group increased to more than twice compared with control (Fig. 3B, 3C, and 3I). Those of mRNA expression level in G1 remarkably increased to several times compared with the control. However, ERα and TGF-βR2 mRNA showed no significant change of mRNA expression level in all groups (Fig. 3H and 3K).

**Measurement of the amount of OCN protein and p38 phosphorylation rate**

We measured the amount of OCN, which is a marker of bone formation, after 20 days incubation with various concentration of genistein. The amount of OCN protein was significantly increased in the genistein added group for 20 days (Fig. 4A). Phosphorylation of p38 was quantified and cultured for 24 h with various concentrations of genistein. All experimental groups showed high p38 phosphorylation activity (Fig. 4B).

**Experiment of direct pulp capping in rats**

We have experimented with direct pulp capping using various concentration of genistein in order to evaluate the tertiary dentin formation and pulp inflammation. In the histological observation, tertiary dentin of the control had porous and rough structure (Fig. 5B, 5C, 5E and 5F). G0.01 and G0.1 were showed high-quality tertiary dentin having dentinal tube structure (Fig. 5H, 5I, 5K and 5L) with odontoblast layer (asterisk), completely covering the surface of the pulp exposure site. But, some tunnel-shaped defects were present in G1 and G10 (Fig. 5N, 5O, 5Q and 5R; arrow). Next, we evaluated that the formation rate and compactness of the tertiary dentin. All genistein applied group indicated higher value in both formation rate and compactness of the tertiary dentin compared with the control. Therefore, we were confirmed genistein applied group became a better in both quantity and quality of the tertiary dentin (Fig. 6A and 6B). In the evaluation of covering degree of pulp exposure site, we have demonstrated that G0.01 and G0.1 was able to improve covering degree compared with the control (Fig. 6C).

Subsequently, we observed the degree of pulp inflammation. We found to be a strong pulp inflammation with capillary expansion and infiltration of inflammatory cells in the control (Fig. 5A and 5D). However, genistein applied group was showed infiltration of inflammatory cells confined to just below the tertiary dentin, inflammatory condition was comparatively mild (Fig. 5G, 5J, 5M and 5P). Moreover, in the evaluation of inflammation pulp, inflammation degree of G0.01 and G0.1 indicated slight inflammation compared to the control (Fig. 6D).

**DISCUSSION**

Genistein is not only used for food containing, also widely known as an inhibitor of TK involved in signal transduction of cell growth or cell proliferation26,27). Genistein is considered that inhibit cell proliferation through regulation of MAPK28-30), and inhibit phosphorylation of ERK and Akt 31-33). The high concentration group (G100) showed suppress the cell proliferation after 3 days incubation. However, in the low concentration genistein addition group (G0.1~10) indicated similar cell proliferation to the control. Effect of genistein as TK inhibitor for RDP was weakly inhibited TK activity in a dose-dependently in case of the low concentration group (G0.1~10). But, the high concentration group (G100) was largely suppressed TK activity. In this study, we used RDP that composed not
only odontoblast precursor cells also contained various cell populations such as fibroblast and vascular cells. We considered that RDP have a different sensitivity to genistein depend on the each cell type, inhibitory effect of TK activity by genistein was less affected in the low concentration group (G0.1~10). However, in the high concentration group (G100), all cells were affected inhibitory effect of TK activity so that cell proliferation was suppressed.

We incubated RDP with genistein indicated
increase of ALPase activity, which is a marker of initial calcification, after 17 days incubation. In the observation of calcified nodule formation by using alizarin red staining, G0.1~G10 showed calcified nodule formation. Especially, the nodule formation of G1 was significantly higher than the other groups. In the results of real time PCR, the expression level of Runx2, DSPP and DMP-1 mRNA increased after 17 days incubation. We quantified the amount of OCN protein by ELISA, increase of OCN has been confirmed after 20 days. mRNA expression level of ERα showed no significantly change. Whereas, ERβ mRNA expression level ascendancy increased by G1~G10. We supposed that these results due to the difference in affinity for ER of genistein. In addition, the expression of ERβ in protein, it was found that the signal at the cell surface has been increased from immunostaining (data not shown). It has been reported that ER expressed in the dental pulp and ERβ has a strong effect. Thus, we considered the low concentration of genistein performed to the differentiation of cells than the inhibitory effects of TK, and the addition of high concentrations of genistein completely inhibits cell proliferation.

To investigate the possibility of new pulp capping agents, we applied genistein to the rat direct pulp capping model. We had selected Ca as a control in vivo experiment. Dentin bridge formation in calcium hydroxide showed faster compared to MTA. In the clinical case, exposed pulp should be closed by the complete dentin bridge in a short period. The formation rate and compactness of tertiary dentin have been improved by genistein compared with the control. But, the results did not change in a concentration-dependent manner. It has been reported that tertiary dentin was induced by direct pulp capping with conventional calcium hydroxide have the tunnel-shaped defects present in approximately 90%, and about 40% of them cause pulpitis. These results suggest quality of the tertiary dentin becomes necessary conditions may not cause degeneration of the pulp and pulp re-infection by leakage through the loss of tertiary dentin. We had investigated the inflammation rate of the pulp...
after operation, low concentration group was minor inflammation compared to the control. It has been reported that genistein may reduce inflammation via inhibition of cyclooxygenase activity\cite{8,9}, and inhibition of vascular permeability by suppressing the expression of VEGF and its receptor gene\cite{10,11}, we considered that suppression of inflammation in these same mechanisms.

So, we supposed that improvement of covering degree is induced by short-term reducing inflammation. Accordingly, genistein use for direct pulp capping agent in the range of optimal concentration (G0.01~G0.1) showed that to induce the high quality tertiary dentin formation, and completely covering of the pulp exposure site, then the pulp inflammations become mild. In this study, we used covalent cross-linked alginic acid sheet for transplantation as a carrier. Now, hydroxyapatite\cite{58}, calcium phosphate\cite{59}, polylactic acid and polyglycolic acid\cite{60,61} have been investigated as a carrier, and these carriers had been proven its efficacy. We regarded that the alginic acid sheet have weak ability to induce hard tissue formation by itself (Fig. 5D and 5E). We supposed that the alginic acid sheet was used as a scaffold for differentiation of odontoblast precursor cells and mesenchymal cells contained in the pulp, through the alginic acid sheet has been developed as a wound covering material. For this reason, the formation of tertiary dentin was observed in only carrier that does not contain genistein, though it is considered that the formation of no defects tertiary dentin was observed by the effect of genistein. However, in our study, genistein applied group take a long time until the formation of tertiary dentin compared with the research that was used cytokines for direct pulp capping agents such as bFGF\cite{62} and BMP2\cite{63}. More studies are needed to make improvements such as change of the carrier in order to shorten the period until the formation of tertiary dentin.

CONCLUSIONS

Our study suggest that genistein was not only inducing calcification in RDP, but also promoting formation of the high quality tertiary dentin in the experiment of direct pulp capping for rats, have a potentiality for new direct pulp capping agent.

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