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Mucus of *Achatina fulica* stimulates mineralization and inflammatory response in dental pulp cells

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Abstract: Dental pulp tissue contains stem cells that can be isolated and used for regenerative medicine in tooth restoration or autologous transplantation. The aim of this study was to observe the mineralization and gene expression in dental pulp cells (DPCs) following treatment with snail mucus. Snail mucus was collected from adult *Achatina fulica* and processed as a dry powder by the freeze-drying technique. The mucus powder was dissolved in a culture medium at a concentration of 15 µg/mL. DPCs were prepared by the outgrowth technique and cultured in a 6-well plate at a density of 5×10^4 cells per well. A mucus-supplemented medium (15 µg/mL) was added to each well. Cell culture was maintained for 3 weeks. The results of Alizarin Red S staining indicated that the DPCs cultured in a medium supplemented with snail mucus showed a higher number of mineralized nodules as compared with the control group cultured in a normal medium. The increased expression of osteopontin and NF-κB reflected the differentiation process of DPCs into bone cells. Snail mucus also induced the expression of some inflammatory genes in DPCs. The results demonstrated that snail mucus has the potential to be used in regenerating and repairing bone and teeth.

Key words: Dental pulp cells, snail mucus, Achatina fulica, mineralization, inflammatory genes

1. Introduction

Dental and oral diseases are major concerns in Thailand, affecting the country's health system. Everyone, at any age, is at risk of losing teeth. Tooth loss can lead to many health problems, including difficulty chewing, which increases the risk of poor nutrition. In addition, tooth loss leaves a large gap, leading to plaque accumulation that can cause periodontal disease. Moreover, the remaining teeth start to shift in an attempt to fill in the gap, leading to misalignment of teeth. Losing teeth may also affect personality, including a loss of confidence that may affect work or social activities. Restoration, with dentures or dental implants, is needed to replace the missing teeth.

Current research has focused on dental and bone implants capable of promoting cell differentiation (Yang et al., 2013; Xavier Acasigua et al., 2014; Naddeo et al., 2015). Cells isolated from tooth pulp contain odontoblasts, fibroblasts, immune cells, and undifferentiated mesenchymal cells called dental pulp stem cells (DPSCs). These findings may lead to restoration using dental pulp cells (DPCs), together with dental implants, to replace lost teeth. Various research groups have proposed the possibility of producing bioactive dental implants by adding natural products, such as natural hydroxyapatite/zircon (Karamian et al., 2014), plant products (Varoni et al., 2012), or herbal extracts (Wang et al., 2012), to dental materials.

Snails have been used in medicine since ancient times. Until the late 19th century, the synthetic peptide ziconotide (SNXIII), which is found in the venom of *Conus magus*, was used as a painkiller following approval by the United States Food and Drug Administration (Bonnemain, 2005). According to reports, snails contain several medically useful substances, including mytimacin-like antimicrobial peptides, which are exclusively found in the mucus of giant African snails (Zhong et al., 2013). In addition, lectin, which acts as an antimicrobial, is also present in the mucus of giant African snails (Ito et al., 2011). High-performance liquid chromatography was used to determine the amount of allantoin and glycolic

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acid in snail mucus and cosmetic creams (El Mubarak et al., 2013). Acharan sulfate, structurally similar to heparin and heparan sulfate, is a glycosaminoglycan found in the mucus of giant African snails (Vieira et al., 2004). For these promising reasons, snail mucus has been used to treat various medical ailments. It has also been observed that snail mucus induces an accumulation of calcium to build and repair a snail shell (Seehabutr, 2008). The calcium crystalline in snails is structurally similar to the type found in bones and teeth, and thus can be developed as materials for engineering applications (Struthers et al., 2002).

Regenerative medicine aims to create dental tissues that can maintain the structure and function of teeth, including repairing damage; stem cell therapy offers potential in the regeneration of bones and teeth to restore the structure and function of lost teeth. Some researchers have suggested the requirements and standards for using cell therapy to treat dental problems (Murakami et al., 2012; Nakashima and Iohara, 2014). Dimitrova-Nakov et al. (2014) indicated that DPSCs can be induced to generate dentin, demonstrating the possibility of using DPCs to repair damaged teeth. However, many problems need to be studied, such as the source of cells that should be used (autologous versus allogeneic stem cells), the mechanism of odontoblast differentiation, and the role of infection and inflammation (Schmalz and Smith, 2014).

This study focuses on the role of inflammatory gene expression and mineralization of DPCs. The main aim of this study is to observe the mineralization with respect to accumulation of calcium in DPCs and define its future role as an effective natural product to treat bone defects.

2. Materials and methods

2.1. Isolation of DPCs

Ethical approval was obtained for this study. This study employed the outgrowth technique from tissue explants. Human dental pulp cells (hDPCs) were freshly derived from a caries-free third molar or premolar that was extracted for orthodontic treatment purposes. The patients gave their written informed consent before enrollment in the study. The gum tissue was removed with a scalpel blade No. 15, and the teeth were wiped with povidone-iodine for 2 min and then wiped with 70% ethanol for 2 min to remove bacteria. The teeth were notched at the dentogingival junction with a chisel before immersion in 15-mL plastic tubes containing complete Dulbecco modified Eagle medium (complete DMEM). The teeth were then washed twice with phosphate-buffered saline (PBS) and once with complete DMEM. The teeth were separated into two parts at the dentogingival junction and a sterile endodontic spoon was used to scoop pulp tissue that was then placed onto a glass dish. The tissue was sliced into small pieces with a sterile blade and cultured in complete DMEM at 37 °C and 5% CO_2. The medium was changed every other day. The DPCs grew out of a piece of tissue for 1–2 weeks. Cells were digested and grown in tissue culture flasks.

2.2. Preparation of snail mucus

The snail mucus samples were collected from adult *Achatina fulica* by mild intermittent irritation in an ultrasonication bath for 5 rounds of 15 s at 30 °C. After each round of mild intermittent irritation, the snails were taken out and the mucus was eluted with 1 mL of distilled water for 1 min. All of the collected mucus was vortex-mixed and concentrated by freeze-drying and kept at –20 °C until use.

2.3. MTT assay

To investigate the cytotoxicity of snail mucus on DPCs, the cultured DPCs (passage 3–4) were seeded into 12-well plates at a density of 2.5×10^4 cells/well. Cells were allowed to adhere for 24 h in complete DMEM at 37 °C and 5% CO_2. The mucus was diluted in complete DMEM to concentrations of 3.75, 7.5, 15, and 30 µg/mL. After the cells were allowed to settle for 24 h, the entire DMEM was discarded and replaced with mucus-supplemented DMEM (0.5 mL/well). The cell culture was maintained at a temperature of 37 °C in a 5% CO_2 incubator for 48 h. The mucus-supplemented DMEM was discarded and replaced with 0.5 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (0.5 mg in 1 mL complete DMEM). The reaction was maintained at 37 °C and 5% CO_2 for 2 h. The MTT-supplemented DMEM was discarded and then 1 mL of DMSO was added. The absorbance of the formazan product was measured at 540 nm and the background was corrected at 630 nm. The percentage of cell viability was compared to that of the control group, which was cultured in complete DMEM. Three replicate assays were performed for statistical analysis.

2.4. Effect of snail mucus on DPCs growth

DPCs (passage 3–4) were seeded into 6-well plates at a density of 5.0×10^4 cells/well. Cells were allowed to adhere for 24 h in complete DMEM at 37 °C and 5% CO_2. DMEM was discarded and replaced with mucus-supplemented DMEM (2 mL/well). The cell culture was maintained at a temperature of 37 °C in a 5% CO_2 incubator for 6 days to allow cell proliferation. The culture medium was changed every 3 days. Cells were trypsinized with 0.25% trypsin/EDTA and counted using a hemocytometer. The amount of cells in the treated group was compared with that of the control group. Three replicate assays were performed for statistical analysis.

2.5. Mineralization assays

DPCs (5×10^4 cells) were seeded into 6-well plates before cultivation in snail mucus-supplemented DMEM (2 mL/well) and maintained at 37 °C in 5% CO_2 for 3 weeks. The culture medium was changed every 3 days. Mineralized

nodules were visualized by staining with Alizarin Red staining. Briefly, the cells were fixed with 4% formaldehyde for 5–10 min and stained with 0.5% Alizarin Red S (pH 4.2) for 5 min. The cells were then rinsed with tap water and viewed with an inverted microscope (Nikon ECLIPSE TS 100). Mineralized nodules were counted and reported as number of nodules per well compared with that of the control group, which was cultured in complete DMEM. Three replicate assays were performed for statistical analysis.

2.6. Gene expression

Cells were cultured for the desired period. The culture medium was discarded and PBS was added to wash the cells. The RNA was then extracted using a High Pure RNA Isolation Kit (Roche). The amount of total RNA was quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific). Genomic DNA was eliminated by adding DNase reaction mixture (Macherey-Nagel) into the total RNA (1 µL DNAse/10 µL RNA) and then the mixture was incubated at 37 °C for 10 min. cDNA was synthesized using iScript Reverse Transcription Supermix and the reverse transcription reaction was performed in an Eppendorf Mastercycler. RT-PCR was performed using a SYBR Green Mastermix (SensiFAST SYBR No-ROX Kit-Bioline) and then analyzed by real-time PCR (LightCycler 480). Relative quantification was performed using LightCycler 480 software 1.5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The list of primers is shown below in the 5' to 3' orientation.

COX-2:	F: CCCTTGGGTGTCAAAGGTAA
	R: GCCCTCGCTTATGATCTGTC
NF-kB:	F: ATGGCTTCTATGAGGCTGAG
	R: GTTGTTGTTGGTCTGGATGC
OPN:	F: GGACAGCCAGGACTCCATTG
	R: TGTGGGGACAACTGGAGTGAA
IL-1β:	F: GCACGATGCACCTGTACGAT
	R: CACCAAGCTTTTTTGCTGTGAGT
IL-6:	F: GGTACATCCTCGACGGCATCT
	R: GCCTCTTTGCTGCTTTCAC
GAPDH:	F: AAGGGCTCATGACCACAGTC
	R: GGATGACCTTGCCCACAG

2.7. Statistical analysis

All studies were conducted at least three times and the results were analyzed by one-way ANOVA, presented as mean ± SD and 95% confidence interval ($P \leq 0.05$).

3. Results

3.1. Cytotoxicity test of snail mucus on DPCs

The MTT results indicated that at the available concentrations of 3.75, 7.5, 15, and 30 µg/mL, snail mucus showed no toxicity to DPCs after 48 h of exposure (Figure 1A). At 48 h, snail mucus had no effect on cell proliferation, because the percentages of cell availability were 105%, 104%, 103%, and 102%, respectively, which were not significantly different from the control group. The concentration of 15 µg/mL was selected for use in the following studies.

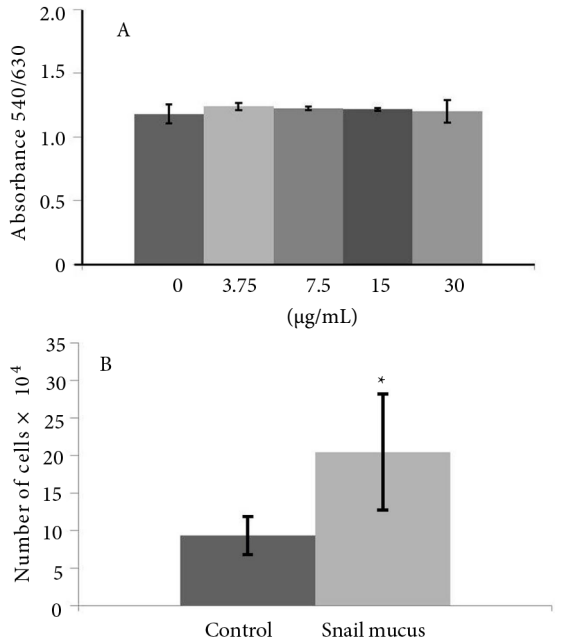

Figure 1. (A) Cytotoxicity test: MTT assay indicates that the snail mucus shows no toxicity and has no effect on cell proliferation of DPCs at 48 h. (B) Effects of snail mucus on DPC growth: at 6 days of culture, the number of cells in the snail mucus-treated group is significantly higher than that in the control group ($P < 0.001$).

3.2. Effect of snail mucus on DPCs growth

When DPCs were treated with 15 µg/mL for 6 days, it was observed that snail mucus affected cell proliferation, because the number of cells was significantly higher than that of the control group (Figure 1B).

3.3. Mineralization assay

Alizarin Red staining indicated that DPCs cultured in mucus-supplemented DMEM (15 µg/mL) for 21 days showed a higher degree of cell mineralization when observed under a microscope (Figures 2A and 2B). The number of mineralized nodules was counted and it was demonstrated that the cells in the mucus-treated group formed more mineralized nodules compared with those of the control group (Figure 2C).

3.4 Expression of osteopontin (OPN) and NF-κB

After culturing for 3 weeks, the expressions of OPN and NF-κB genes were investigated by reverse transcription and RT-PCR. An increased expression of the OPN gene was observed in the mucus-supplemented group as compared with the control group (Figure 3). Although

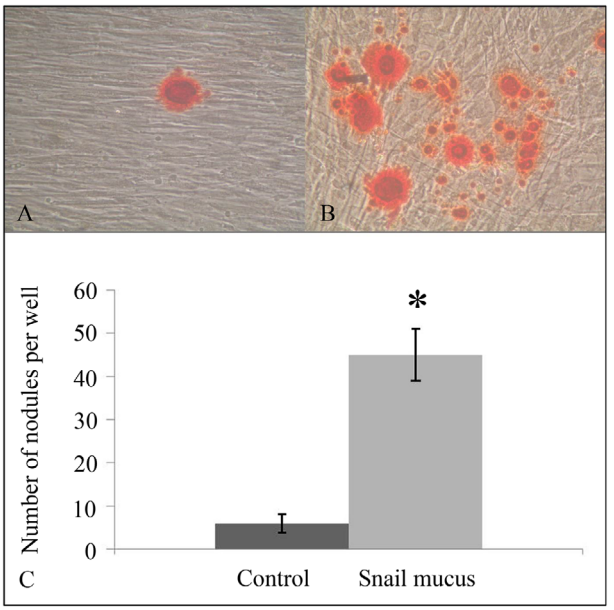

Figure 2. Mineralization of DPCs: at 3 weeks of cell culture, the DPCs supplemented with snail mucus (B) showed a higher degree of mineralization as compared with the control group (A). The DPCs supplemented with snail mucus showed a significantly higher number of mineralization nodules as compared with the control group ($P < 0.001$) (C).

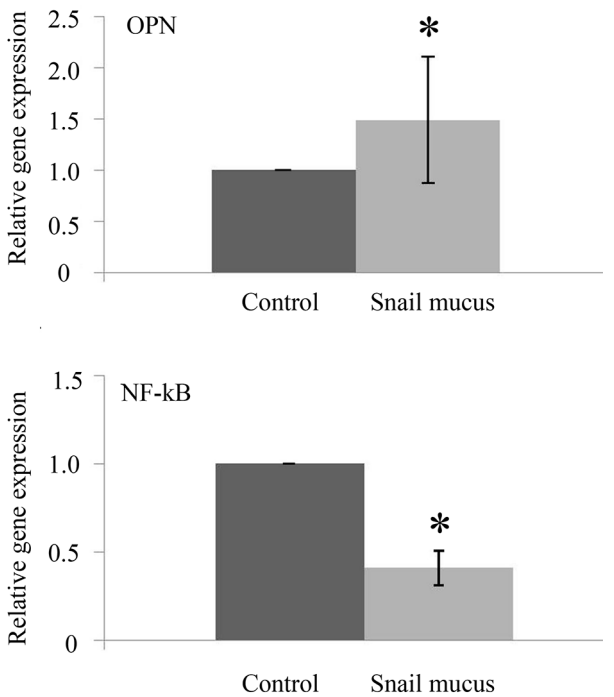

Figure 3. Expression of OPN: increased expression of the OPN gene (*$P < 0.012$) and a decreased expression of NF-κB (*$P < 0.0001$) were observed in DPCs cultured in complete DMEM supplemented with snail mucus for 3 weeks as compared with the control group.

gene expression was statistically significant, the fold change of the gene was less than 2-fold. A decreased expression of the NF-κB gene was observed in the mucus-supplemented culture as compared with the control group (Figure 3).

3.5. Expression of inflammatory genes

To determine the effects of snail mucus on the expression of cytokines and the inflammatory mediator in DPCs following 3 weeks of culture, the expressions of three inflammatory genes, interleukin-6 (IL-6), interleukin-1β (IL-1β), and cyclooxygenase-2 (COX-2), were investigated by reverse transcription and RT-PCR. An increased expression of inflammatory genes was observed in the mucus-supplemented culture as compared with the control group (Figure 4).

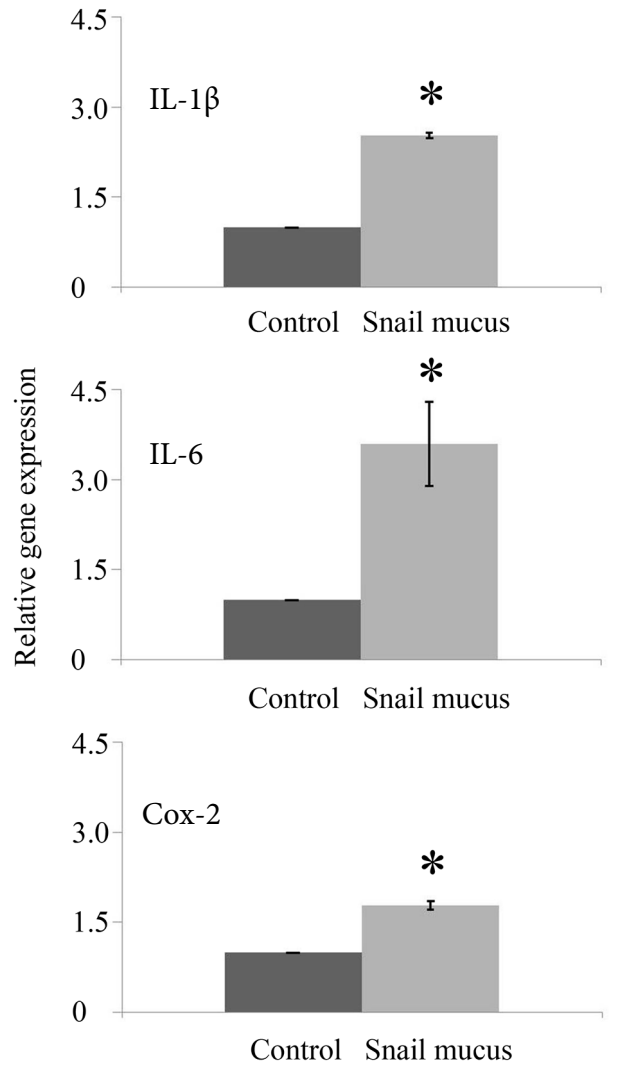

Figure 4. Expression of inflammatory genes: increased expression of IL-1β, IL-6, and COX-2 was observed in DPCs cultured in complete DMEM supplemented with snail mucus for 3 weeks as compared with the control group (*$P < 0.0001$).

4. Discussion

Previous studies have indicated that tooth pulp is the source of mesenchymal stem cells (MSCs) (Feng et al., 2014; Hakki et al., 2015). These kinds of cells, also known as DPSCs, can be used in tissue engineering (Sanz et al., 2015). Earlier researchers showed that stem cells from tooth pulp can develop into bone and this bone formation activity may be useful for osseointegrated dental implants (Yamada et al., 2010). DPSCs are a type of MSCs characterized by self-renewal and multilineage differentiation, making them an attractive choice for tissue engineering purposes, which may provide a foundation for autologous transplantation of DPSCs (Feng et al., 2013a, 2013b).

The present study used cells isolated from dental pulp tissue, which contains odontoblasts, fibroblasts, and undifferentiated mesenchymal cells (Yu and Abbott, 2007). The use of hDPCs from dental pulp tissue, which comprises a heterogeneous cell population, allowed us to observe the response of all cell types in the pulp cavity, as if the bone prosthesis composite mucus would be applied in vivo. This study investigated changes in gene expressions related to mineralization when snail mucus was applied to hDPCs in vitro. The experimental results indicated that snail mucus promoted mineralization in DPCs. The increased OPN gene expression confirmed that snail mucus promoted osteoinduction in DPCs. However, the fold change of OPN expression was not high; it is possible that the increased expression occurred only in DPSCs, which is a small population within the DPCs (mostly composed of fibroblasts). Many earlier studies reported the negative roles of NF-kB in odonto-/osteogenic differentiation and mineralization (Liu et al., 2010, 2013; Li et al., 2014). Thus, the decreased expression of NF-κB in the present study was consistent with these previous studies. Additionally, a recent study by Hozhabri et al. (2015) reported that a decrease in NF-κB in DPSCs in the presence of inflammatory cytokines enhanced odontoblastic differentiation and collagen matrix formation. This evidence indicates that using the mucus of African snails may offer promise in the regeneration of bones and teeth.

Bone healing commences with an inflammatory reaction that initiates the regenerative healing process, leading in the end to reconstitution of the bone itself (Schmidt-Bleek et al., 2012). The inflammatory phase, the first stage of bone healing, occurs during the first week, followed by the reparative stage, which begins about 2 weeks after the fracture occurs.

Increased levels of IL-6 and COX-2 have been detected in inflamed pulp tissue with the involvement of dental pulp fibroblasts in the development of pulpitis (Lin et al., 2002). Naik et al. (2009) mentioned that expression of COX-2 during the early inflammatory phase of repair regulates chondrogenesis, bone formation, and remodeling. Huang et al. (2014) reported that COX-2-deficient periosteal progenitors present impaired osteogenic and chondrogenic differentiation in cell culture. In the present study, mucus induced inflammatory COX-2 expression in the DPCs. Further studies are required to clarify whether the expression of the COX-2 gene occurs in dental pulp fibroblast or in DPSCs. If COX-2 is dominantly expressed in dental pulp fibroblasts, it may be necessary to reduce the inflammatory reaction or pain response after mucus is applied in vivo.

IL-1β is a proinflammatory cytokine that negatively affects the suppression of bone mineralization (Lacey et al., 2009). Chien et al. (1999) showed that IL-1β inhibited the formation of mineralized tissue nodules by periodontal ligament cells (Chien et al., 1999). IL-1β inhibited RUNX2 and collagen expression in osteoblasts (Ding et al., 2009).

Many cell types in bone, including osteoblasts, mononuclear phagocytes, endothelial cells, chondrocytes, fibroblasts, and keratinocytes, can produce IL-6 (Littlewood et al., 1991; Yang et al., 2007), which transiently indicates that the role of IL-6 appears to be most important in the early stages of fracture healing, because IL-6–knockout mice showed delayed callus maturity, mineralization, and remodeling compared with the callus of the wild-type mice (Yang et al., 2007). IL-6 signaling, therefore, appears to play a role in the early stages of fracture healing, but its role diminishes over time (Yang et al., 2007). An earlier study showed that IL-6 expressed in hDPCs following stimulation with lipopolysaccharide (Tokuda et al., 2001).

Snail mucus appears to affect mineralization and slightly induces inflammation in DPCs. As the inflammation process is part of cell differentiation and mineralization, whether the dynamic changes of gene expression occur in response to stem cell differentiation or to the inflammatory response of dental pulp fibroblasts needs to be investigated. This phenomenon is important in the application of snail mucus for regenerating bones and teeth, because the inflammation of dental pulp fibroblasts may decrease bone formation. Since the expression of the OPN gene is not high enough to confirm osteoinduction, further studies with other osteogenic markers are required as well as a time course study.

In conclusion, snail mucus from *Achatina fulica* promoted mineralization in DPCs as confirmed by the upregulation of OPN. A decreasing NF-κB expression level revealed its negative role in odontoblastic differentiation of DPCs. However, the expression of some inflammatory genes should be a concern, because the proinflammatory cytokines (IL-1β and IL-6) and the inflammatory

mediator (COX-2) play dual roles in bone differentiation and mineralization. The effect of snail mucus on the inflammation of DPCs needs further investigation and stem cells should be isolated.

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