

# 담즙산이 *Porphyromonas gingivalis* 리포다당질에 의해 유도된 염증반응에 미치는 효과

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## The effect of bile acids on *Porphyromonas gingivalis* lipopolysaccharide-induced inflammatory response

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**Objectives:** In this study, I aimed to evaluate the inhibitory effect of bile acids on the inflammatory response and osteoclastogenesis in RAW 264.7 cells activated through lipopolysaccharide (LPS) and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) of the periodontal pathogen *Porphyromonas gingivalis*.**Methods:** Myelomonocytic RAW 264.7 cells were activated through *P. gingivalis* LPS to induce inflammatory response, and were treated with three bile acids, including taurodeoxycholate, taurocholate, and glycocholate at different concentrations. The cytotoxicity of bile acids was assessed through the MTT assay. To evaluate the inhibitory effect of bile acids on inflammatory response, the induction levels of the pro-inflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  were measured using ELISA 12 h after the treatment. Additionally, after activating the cells with RANKL to promote osteoclastogenesis, we examined whether bile acids suppressed osteoclast differentiation using the tartrate-resistant acid phosphatase staining.**Results:** In the cell viability test, taurodeoxycholate and taurocholate did not exhibit any cytotoxic effect on RAW 264.7 cells at concentrations equal to or less than 200  $\mu$ M, and glycocholate was non-cytotoxic until the maximal concentration (4,000  $\mu$ M). All the three bile acids exhibited an inhibitory effect on inflammatory response, as the production levels of pro-inflammatory cytokines, including IL-6 and TNF- $\alpha$ , decreased with an increase in the concentration of the three bile acids in a dose-dependent manner. The expression of IL-6 reduced remarkably upon treatment with taurodeoxycholate and glycocholate ( $P < 0.001$ ), while the expression of TNF- $\alpha$  decreased slightly upon treatment with glycocholate ( $P < 0.05$ ). Moreover, only glycocholate at a concentration of 1,000  $\mu$ M suppressed osteoclast differentiation of RAW 264.7 cells ( $P < 0.001$ ), while taurodeoxycholate and taurocholate did not exhibit an inhibitory effect on osteoclastogenesis.**Conclusions:** Here, I showed that all the three bile acids (taurodeoxycholate, taurocholate, and glycocholate) inhibited *P. gingivalis* LPS-induced inflammatory response, and glycocholate partially suppressed RANKL-mediated osteoclastogenesis in RAW 264.7 cells.**Key Words:** Bile acids, Glycocholic acid, Inflammation, Periodontitis, *Porphyromonas gingivalis*, Taurodeoxycholic acid

## Introduction

Periodontitis is involved with oral microorganisms, environmental conditions, and host factors. The host-immune system often responds to microbial or environmental factors to aggregate inflammatory cells and increase the expression of pro-inflammatory cytokine such as interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$ , proteolytic enzymes, and bone metabolism-related factors, finally resulting in destruction of periodontal tissue<sup>1-3</sup>.

Especially, gram-negative anaerobes in biofilms such as *Porphyromonas gingivalis* and its lipopolysaccharide (LPS) play an important role in the etiopathogenesis of periodontitis. *P. gingivalis* has a variety of virulence factors and induces the secretion of pro-inflammatory cytokines in periodontal tissue, thereby activating a receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and osteoclast expression and resulting in alveolar bone destruction<sup>1-3</sup>. In recent years, peri-implant diseases with similar characteristics to periodontal diseases have also increased, the periodontopathogens closely related to periodontitis such as *P. gingivalis* and *Treponema denticola* were also found at high levels in inflamed peri-implant sites<sup>4,5</sup>.

The periodontal pathogens and its metabolites in periodontal tissue could impact on systemic conditions<sup>6-8</sup>. Many studies have suggested that periodontal disease and several chronic diseases are correlated through the action of common risk factors. Comprehensively, chronic inflammation can be described as a shared mechanism of linking periodontal diseases including periodontitis and peri-implantitis, and various systemic diseases<sup>9-12</sup>. Therefore, the control of the inflammatory process is an essential target for the management of both periodontal and systemic diseases.

These days, inflammation has become more important as a critical factor in the pathogenesis of many systemic diseases. Many studies have studied on substances capable of regulating the immune-inflammatory response and the accompanying tissue-destructive metabolism. In particular, the anti-inflammatory effects of well-known natural substances, foods, and substances derived therefrom are evaluated. In dentistry, it is noted whether the anti-inflammatory components can also inhibit periodontal inflammation and breakdown such as bone resorption<sup>2,13-15</sup>. For example, it was confirmed that milk and dairy products inhibited expression of pro-inflammatory cytokines in gingival fibroblast as well as intestinal inflammation<sup>15</sup>. The anti-inflammatory action of food-derived antioxidants or commonly used antibiotics also had the impact on inflammatory process induced by *P. gingivalis* LPS<sup>2,13,14</sup>.

Bile acids or bile salts as a major component of bile juice emulsify fat to help lipolysis and form an osmotic gradient to promote bile secretion. Glycocholate and taurocholate are the primary bile acids, and secondary bile acids are sometimes produced by gut microbiota<sup>16</sup>. Bile and related extracts (eg, bear bile) are used as a cholagogue (choleretic) or improvement of liver function in clinical use. In addition, several recent studies reported the potentials of bile acids as a regulator for inflammation. Bile acids can bind to specific receptors to regulate inflammation<sup>17</sup>, and help inhibiting various inflammatory diseases<sup>18-24</sup>.

One of bile acids, glycocholate, presented an inhibitory effect on inflammatory activation induced by *E. coli* LPS. It suppressed the activations of dendritic cells and subsequent T cells by LPS and also reduced the expressions of TNF- $\alpha$  and IL-12p40 in murine model<sup>18</sup>. In another study, taurocholate also decreased the levels of IL-1 $\beta$ , interferon- $\gamma$  and TNF- $\alpha$  in colon tissues and alleviated inflammatory signs on ulcerative colitis<sup>21</sup>. Secondary bile acid, taurodeoxycholate was also examined as a therapeutic agent to prevent sepsis and atopic dermatitis. Its intravenous infusion suppressed septic response triggered with LPS and alleviated abnormal inflammatory reactions on cutaneous tissues<sup>19,20</sup>. Taurodeoxycholate also had anti-inflammatory effect on stomach and promoted the proliferation of intestinal cells and inhibited the apoptosis induced by the activation of NF- $\kappa$ B<sup>23,24</sup>.

The anti-inflammatory action of bile acids in other tissues can also be involved in the inflammatory mechanism of periodontal tissue. The regulation of inflammation is both locally and universally important as considering the inflammation as a link between periodontitis and systemic diseases. However, no studies have evaluated whether bile acids can control periodontal inflammation and its accompanying tissue-destructive processes. Therefore, it is necessary to investigate the potential of these universal inflammatory regulators exert on process of periodontitis. Therefore, this study aimed to evaluate the inhibitory effect of bile acids on the inflammatory response and osteoclastogenesis in macrophage cell line RAW 264.7 stimulated by periodontopathogens *P. gingivalis*-LPS and RANKL.

## Materials and Methods

### 1. Bile acids and reagents

Three bile acids (bile salts) were selected as experimental substances. They are sodium taurodeoxycholate (Sigma, St. Louis, MO, USA), sodium taurocholate (Alfa Aesar, Haverhill, MA; Sigma), and sodium glycocholate (Sigma). *P. gingivalis* 33277

LPS (Pg-LPS; InvivoGen, San Diego, CA, USA), the macrophage colony-stimulating factor (M-CSF; R&D systems, MN, USA), and recombinant truncated mouse RANKL (R&D systems) were used as cell stimulators.

## 2. RAW 264.7 cell cultures

Murine cell-line RAW 264.7 (ATCC® TIB-71TM; American Type Culture Collection, Manassas, VA, USA; RRID:CVCL\_0493) were used. This myelomonocytic cell line can differentiate into macrophages which are immune cells regulating inflammatory response or into osteoclasts depending on surrounding regulatory factors such as M-CSF, RANKL, and physiological environment.

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) with high glucose (4,500 mg/L), supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma). They were incubated in 100 mm culture dishes (Corning, Corning, NY, USA) under 37°C, 5% CO<sub>2</sub>, and humidified conditions.

Adherent cells in culture dishes were collected by a cell scraper after culturing in 1-3 days and seeded in 6, 24, and 96-well plates (Corning and SPL, Pocheon, Korea). The cells were activated by LPS (1 µg/ml) from the *P. gingivalis* strain to induce inflammatory responses similar to periodontitis, and stimulated by M-CSF (30 ng/mL) and RANKL (100 ng/mL) to promote osteoclastogenesis. Three bile acids were treated at different concentrations (0-4,000 µM) in the absence or presence of Pg-LPS, M-CSF, and RANKL. The treatment and control cells were incubated for different hours according to experiments.

## 3. Cell viability test by MTT-assay

Cell viability or cell cytotoxicity was assessed by MTT-assay. RAW 264.7 cells were seeded at a density of  $3 \times 10^3$  cells/well onto 96-well plates and incubated in a 37°C and 5% CO<sub>2</sub> for 24 hours. The wells were treated then with either only culture medium 100 µl (control) or mixture of three bile salts at different concentrations with or without Pg-LPS (1 µg/ml); sodium taurodeoxycholate and sodium taurocholate were treated at the range of 0-1,000 µM (0, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 µM), and sodium glycocholate at the range of 0-4,000 µM (0, 20, 50, 100, 200, 500, 1,000, 2,000, and 4,000 µM), each. After 24 hours incubating, 20 µl of 5 mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Alfa Aesar) solution was added in each well. The plates were incubated for 3 hours further for the formation of MTT formazan, which reflects the amounts of living cells. After the removal of

the supernatant and adding of 100 µl DMSO solution, the optical density was measured at 570 nm on a microplate reader.

## 4. Cytokine detection by ELISA

RAW 264.7 cells were cultured at a density of  $3 \times 10^4$  cells/well in 6-well plates in a 37°C and 5% CO<sub>2</sub> for 24 hours. The wells were treated then with either culture medium alone (control) or three bile salts at serial concentrations under the stimulation of Pg-LPS (1 µg/ml). According to the results of cell viability test, sodium taurodeoxycholate and sodium taurocholate were treated in the range of 0-100 µM (0, 1, 10, and 100 µM) and sodium glycocholate were done in the range of 0-1,000 µM (0, 1, 10, 100, and 1,000 µM), as the limited concentration without cell toxicity. Culture supernatants were collected after incubating for 12 hours (the treatment time was determined by preliminary experiments done with several time points), and stored at -80°C.

The levels of pro-inflammatory cytokines IL-6 and TNF-α in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). ELISA kit (Ready-SET-Go; eBioscience Affymetrix, San Diego, CA, USA) and related reagents were used in accordance with the manufacturer's instructions. After reagent treatments, the optical density of the plates was measured at 450 nm.

## 5. Osteoclastogenesis detection by TRAP staining

$2 \times 10^3$  cells/well of RAW 264.7 cells were seeded in 96-well plates and treated with Pg-LPS (1 µg/ml) and/or RANKL (100 ng/mL) combined with M-CSF (30 ng/mL). Three bile salts were added in the range of 0-1,000 µM (0, 1, 10, 100, and 1,000 µM). After 3 days incubating, the culture medium was exchanged by fresh control or reagent-treated ones, and the cells were allowed to differentiate for 1 day more. After total 4 days of culturing, the cells were fixed with 3.7% paraformaldehyde (Alfa Aesar) for 15 minutes and permeabilized by 0.1% Triton X-100 (Sigma) for 1 minute.

Tartrate-resistant acid phosphatase (TRAP) staining was carried out with TRAP kit (Leukocyte Acid Phosphatase kit; Sigma) according to the manufacturer's instruction. TRAP-positive multinucleated cells (with  $\geq 3$  nuclei) were regarded as osteoclast or osteoclast-like cells (OLCs), which were counted under a digital inverted light microscope with the camera (Nikon, Tokyo, Japan).

## 6. Statistical analysis

Results were presented as the mean and standard deviation (S.D). One-way analysis of variance (ANOVA) followed by Tukey

test was used in the statistical analyses and  $P$ -values  $<0.05$  were considered statistically significant. All statistical analyses and graphics were performed by Graphpad Prism version 5.01 software (Graphpad software Inc., Graphpad San Diego, CA, USA, RRID:SCR\_002798).

## Results

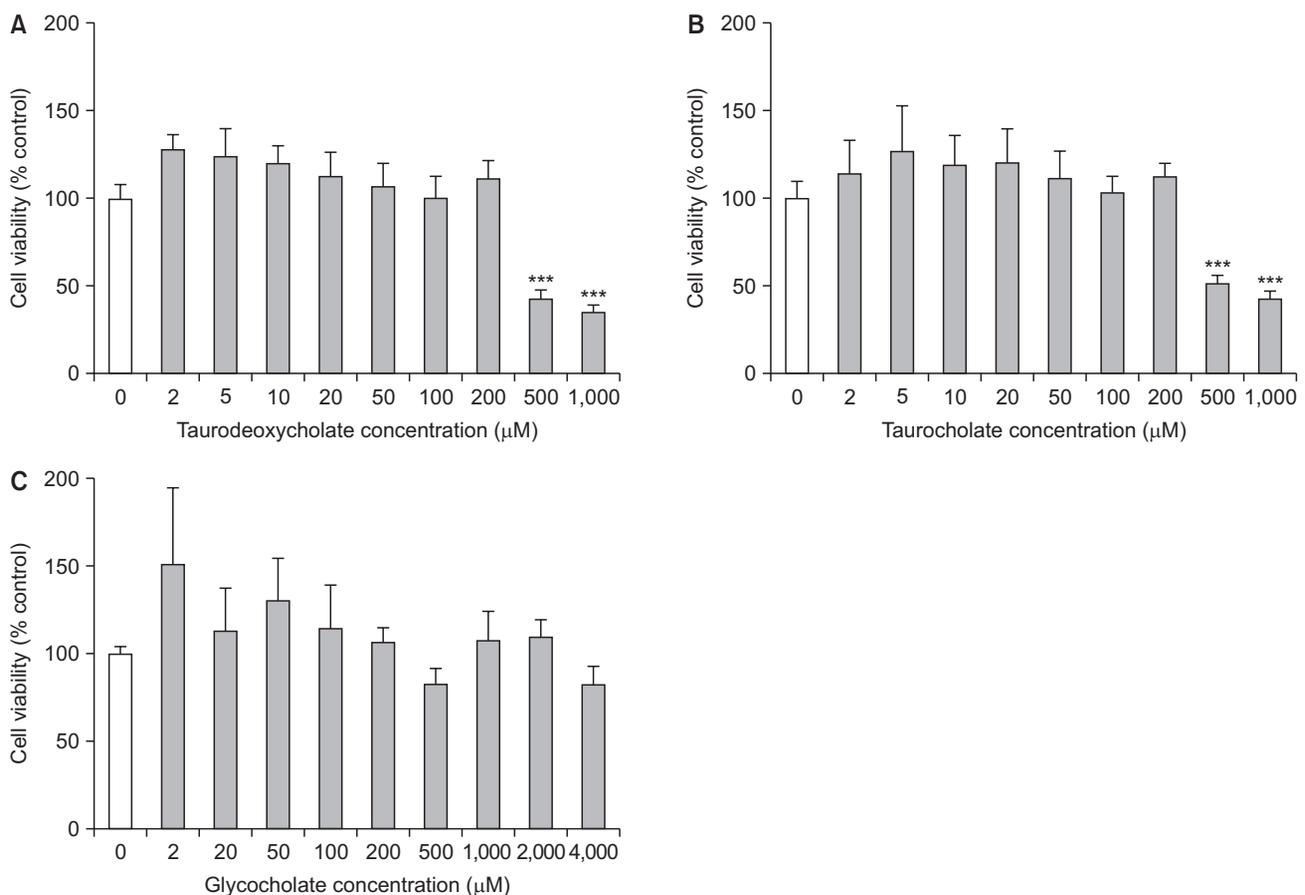
### 1. Three bile acids had no cytotoxic effect at most concentrations on RAW 264.7 cells

RAW 264.7 cells were treated with culture medium alone or in combination with bile salts, and then cell viability was measured after 24 hours by MTT-assay. In results, cell viabilities of three bile salts-treated groups were not significantly different compared to that of the control group at most concentrations (Fig. 1). The cell viabilities of sodium taurodeoxycholate and

sodium taurocholate were similar to or higher than that of the control group at the concentration of less than 200  $\mu\text{M}$ . However, both compounds presented cytotoxicity to the cells 500 and 1,000  $\mu\text{M}$  ( $P < 0.001$ ) (Fig. 1A, B). Meanwhile, sodium glycocholate presented nontoxic effect up to 4,000  $\mu\text{M}$ , a maximum concentration of the experiment (Fig. 1C).

### 2. Three bile acids inhibited the production of pro-inflammatory cytokines IL-6 and TNF- $\alpha$ in Pg-LPS-stimulated RAW 264.7 cells

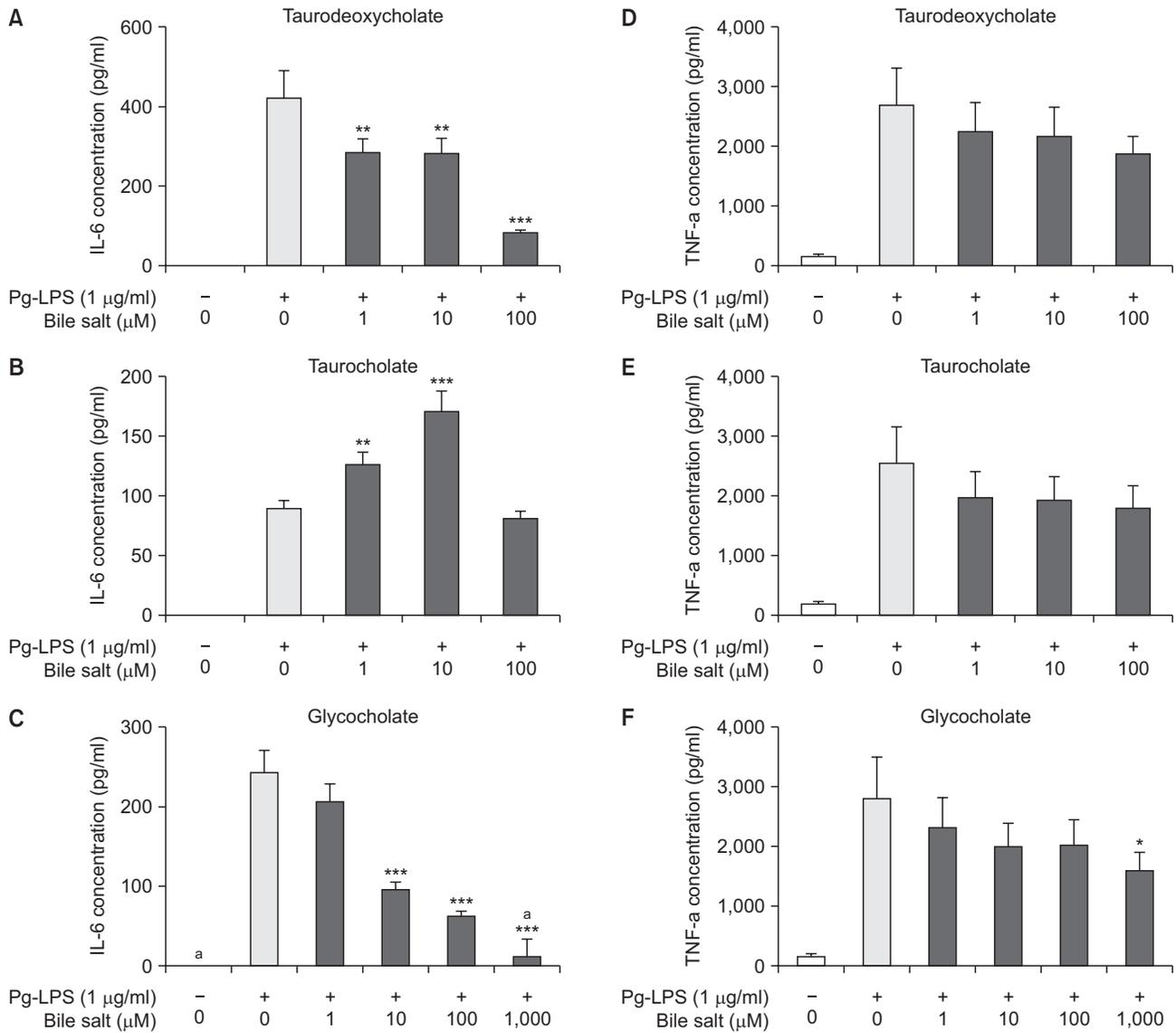
To evaluate the effect of three bile acids on the inflammatory response, the induction levels of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$  after treatment of the compounds during 12 hours were measured by ELISA. The production of IL-6 and TNF- $\alpha$  increased significantly in the cell supernatants stimulated under Pg-LPS alone compared to the control ones,



**Fig. 1.** Effect of bile acids on the viability of RAW 264.7 cells. RAW 264.7 cells ( $3 \times 10^3$  cells/well) were seeded in 96 well-plates and treated with culture medium alone (control) or in combination with bile salts. After 24 hours, the cell viabilities represented as optical density at 570 nm were measured by MTT-assay. (A, B) Sodium taurodeoxycholate and sodium taurocholate were treated at the range of 0–1,000  $\mu\text{M}$ . The cell viabilities of them were no significant differences with that of the control group. However, both groups presented cytotoxicity to the cells 500 and 1,000  $\mu\text{M}$  ( $P < 0.001$ ). (C) Sodium glycocholate treated at the range of 0–4,000  $\mu\text{M}$  presented no cytotoxic effect up to a maximal 4,000  $\mu\text{M}$ . Results were expressed as the relative cell viabilities (%) compared to the control group (no bile salt: 0  $\mu\text{M}$ ) as a reference. Bar and error bar present the mean  $\pm$  S.D. of % cell viability relative to the control. \*\*\* $P < 0.001$  significantly different from the control. Each point is repeatedly measured.

while the expression of both cytokines tended to be suppressed with the increasing of concentration of three bile salts in a dose-dependent manner (Fig. 2). Notably, the production of IL-6 was strongly inhibited at concentration higher than 100  $\mu\text{M}$  of sodium taurodeoxycholate or 10  $\mu\text{M}$  of sodium glycochol-

ate, each ( $P < 0.001$ ) (Fig. 2A, C). The induction of TNF- $\alpha$  was also suppressed as the concentration of each bile salt increased, however, the significance of the trend was relatively weak compared to that of IL-6. The levels of TNF- $\alpha$  were significantly lowered only in the treatment of 1,000  $\mu\text{M}$  of sodium glycochol-



**Fig. 2.** Effects of three bile acids on expression of pro-inflammatory cytokines in Pg-LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were cultured at a density of  $3 \times 10^4$  cells/well in 6-well plates and treated with either culture medium alone (control) and three bile salts with the serial concentrations in the range of 0–100  $\mu\text{M}$  or 0–1,000  $\mu\text{M}$  under the stimulation of Pg-LPS (1  $\mu\text{g/ml}$ ) for 12 hours. The levels of pro-inflammatory cytokines IL-6 and TNF- $\alpha$  in the culture supernatants were measured by ELISA. The production of IL-6 (A–C) and TNF- $\alpha$  (D–F) increased significantly under Pg-LPS stimulation alone compared to the control, while the expression of them tended to be decreased with increasing treated concentration of three bile salts in a dose-dependent manner compared to only Pg-LPS group. (A–C) The production of IL-6 was strongly inhibited at concentrations equal to or higher than 100  $\mu\text{M}$  of sodium taurodeoxycholate or 10  $\mu\text{M}$  of sodium glycocholate treatment, each ( $P < 0.001$ ). (D–F) The production of TNF- $\alpha$  was suppressed as the concentration of three bile salts increased although the significance was relatively weak than IL-6. The levels of TNF- $\alpha$  were significantly lowered only in the treatment of sodium glycocholate at 1,000  $\mu\text{M}$  ( $P < 0.05$ ). Results (bar and error bar) present the mean  $\pm$  S.D. of cytokine levels (pg/ml). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  mean significant differences from Pg-LPS only group. 'a' means no significant differences with the control (no Pg-LPS) group. Each point is repeatedly measured ( $n = 6$  well in treatment,  $n = 3$  in ELISA). Pg-LPS, *Porphyromonas gingivalis* lipopolysaccharide; IL-6, interleukin-6; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ELISA, enzyme-linked immunosorbent assay.

ate ( $P<0.05$ ) (Fig. 2D-F).

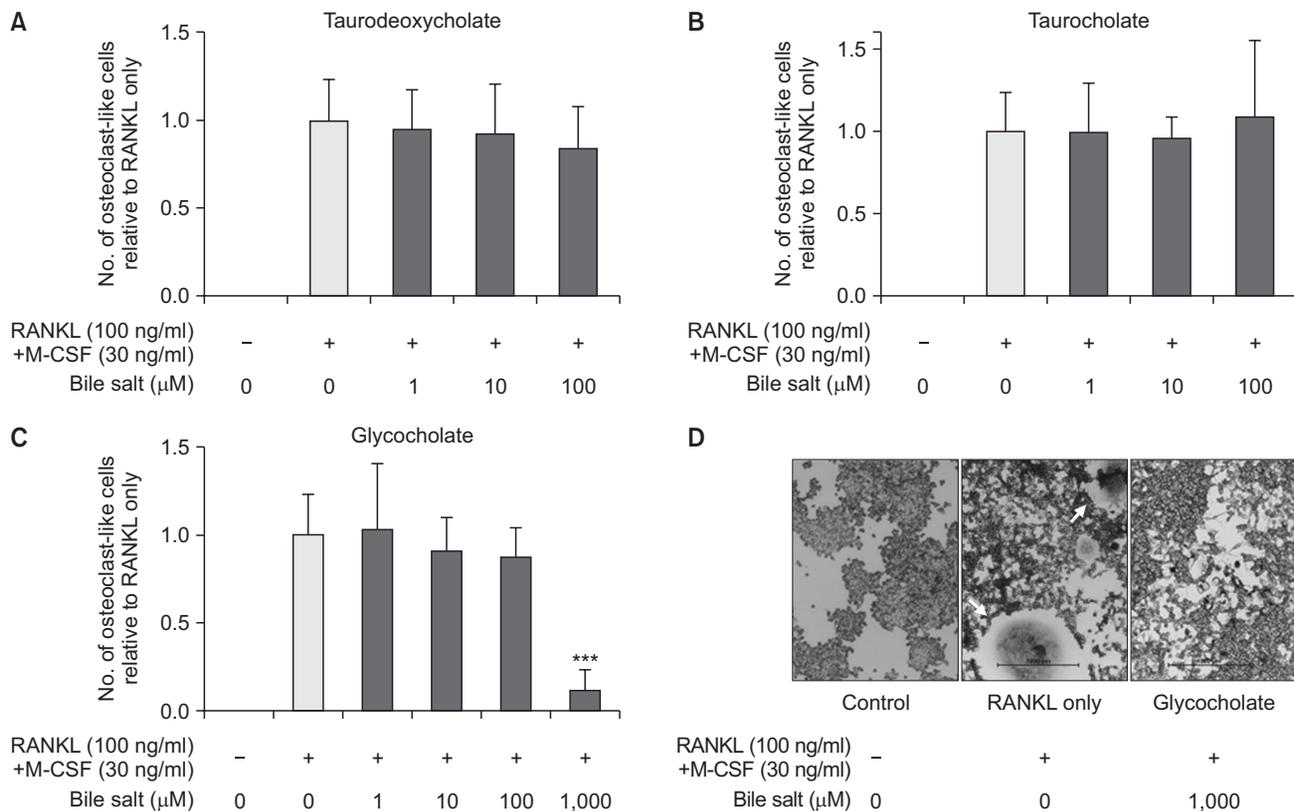
### 3. Sodium glycocholate at the highest concentration remarkably suppressed osteoclast differentiation of RAW 264.7 cells

To evaluate the impact of bile acids to osteoclastogenesis induced by Pg-LPS, RANKL, and M-CSF in RAW 264.7 cells, the cells treated with the stimulators and bile salts for 4 days were stained by TRAP method. TRAP-positive multinucleated cells (with  $\geq 3$  nuclei) were regarded as osteoclast or OLCs. In this experiment, RAW 264.7 cells stimulated by Pg-LPS were not differentiated to the TRAP-positive cells. On the other hand, in the groups activated by RANKL with M-CSF, the cells were differentiated to multinucleated giant cells and stained TRAP-

positively. However, the amounts of osteoclast or OLCs in the groups added with bile acids were no significant difference compared to those of only RANKL and M-CSF group, except a part of the treatment of sodium glycocholate (Fig. 3A-C). The sodium glycocholate at the highest concentration of 1,000  $\mu\text{M}$  remarkably suppressed osteoclast differentiation of RAW 264.7 cells ( $P<0.001$ ) (Fig. 3C, D), while sodium taurodeoxycholate and sodium taurocholate were no significant effect on the inhibition of osteoclastogenesis (Fig. 3A, B).

## Discussion

This study aimed to examine the inhibitory effect of bile acids on the inflammatory response and osteoclastogenesis in my-



**Fig. 3.** Effects of three bile acids on osteoclast differentiation of RAW 264.7 cells stimulated by RANKL. (A-C) RAW 264.7 cells ( $2 \times 10^3$  cells/well) seeded in 96-well plates and treated with RANKL (100 ng/ml) combined with M-CSF (30 ng/ml) in the absence or presence of three bile salts in the range of 0–100 or 0–1,000  $\mu\text{M}$ . After 4 days, the fixing of cells and TRAP staining was carried out. TRAP-positive multinucleated cells (with  $\geq 3$  nuclei) were regarded as osteoclast or OLCs. The treatment of RANKL and M-CSF (only RANKL group) stimulated the cells to differentiate to multinucleated cells as osteoclast or OLCs. However, the bile salts treatments did not suppress osteoclast differentiation of the cells at most concentrations. (A, B) While sodium taurodeoxycholate and sodium taurocholate were no effect on the inhibition of osteoclastogenesis, (C) sodium glycocholate at 1,000  $\mu\text{M}$  (the highest concentration) significantly suppressed osteoclast differentiation of RAW 264.7 cells ( $P<0.001$ ) (D) as showed in the pictures of TRAP staining for sodium glycocholate treatment group. The RANKL only group presented many multinucleated giant cells as the cells indicated red arrows, while the cells with 1,000  $\mu\text{M}$  sodium glycocholate were similar to ones of the control. Results were expressed by relative number (ratio) of TRAP positive-OLCs to the only RANKL group. Bar and error bar present the mean  $\pm$  S.D. of relative ratio to the only RANKL without bile salt as a reference value (1.00). \*\*\* $P<0.001$  significantly different from the only RANKL group. Each point is repeatedly measured ( $n=4$ ). RANKL, activating receptor activator of nuclear factor- $\kappa\text{B}$  ligand; M-CSF, macrophage colony-stimulating factor; TRAP, tartrate-resistant acid phosphatase; OLCs, osteoclast-like cells.

elomonocytic cells RAW 264.7 stimulated by periodontopathogen *P. gingivalis*-LPS and RANKL. The study assessed the effect of bile acids on the cells related to both immune and bone metabolisms as considering periodontal inflammation. RAW 264.7 cells were used because they could differentiate macrophage related inflammation or osteolytic cells. Although several studies evaluated the effect of bile acids on other immune cells and inflammatory disease models such as atopic dermatitis, ulcerative colitis, and sepsis, there was no study to examine the effect on periodontitis or osteoclast differentiation. This study first evaluated osteoclast differentiation because the process of bone loss accompanied by an inflammatory response is critical at periodontitis.

In the test for the cytotoxic effect of bile salts on RAW 264.7 cells, there is no toxic effect to the cells at the most concentration; sodium taurodeoxycholate and sodium taurocholate presented non-cytotoxic at the concentration of equal to or less than 200  $\mu\text{M}$ , while sodium glycocholate did at the maximal concentration of 4,000  $\mu\text{M}$ . Thus, the sodium glycocholate can be applied in a broader range of concentration. The results correspond to the previous study reporting that glycocholate had the anti-inflammatory effect without cytotoxicity within a range of 0.01-1 mg/ml (about 20-2,000  $\mu\text{M}$ ) on other immune cells and the most appropriate concentration of taurodeoxycholate applied to bone marrow-derived immune cells was 0.05 mg/ml (about 100  $\mu\text{M}$ )<sup>18</sup>. On the other hand, taurodeoxycholate at 500-1,000  $\mu\text{M}$  had no toxic effect to intestinal epithelial cell lines and rather promoted their proliferation in another study<sup>23</sup>. It may be due to differences in the types of cells (immune cells or epithelial cells) and their cell signal mechanisms.

To assess the inhibition of three bile acids to inflammatory responses, the levels of the released pro-inflammatory cytokines IL-6 as well as TNF- $\alpha$  were measured in the conditions combined with Pg-LPS and three bile salts. The expression of IL-6 decreased as the concentration of bile salts increases, and particularly did significantly in sodium taurodeoxycholate and sodium glycocholate. Thus, this study confirmed these bile acids had the inhibitory effect on IL-6 in the immune cells stimulated by a periodontopathogen Pg-LPS. Several studies have reported these bile acids had the anti-inflammatory effect of reducing TNF- $\alpha$  and IL-1 $\beta$  in other tissues including inflamed colon tissue, cutaneous tissue as well as immune cells under another type of LPS stimulation<sup>18,19,21</sup>. The study also presented a similar anti-inflammatory effect on myelomonocytic immune cells activated by Pg-LPS. However, the effect was stronger to IL-6 than TNF- $\alpha$ . The inhibition levels of TNF- $\alpha$  correspond the study with primary macrophages, where the treatment of glycocholate

at 1,000  $\mu\text{M}$  decreased the expression levels of TNF- $\alpha$  to about 50% compared to the control<sup>18</sup>. Meanwhile, taurodeoxycholate at 100  $\mu\text{M}$ , which was suggested as the optimal concentration in another study, had a weak effect to TNF- $\alpha$  in this study<sup>18</sup>. Considering the concentration with non-cytotoxic effect, taurodeoxycholate and especially glycocholate are effective to the inhibition of inflammatory mediators.

This study also first examined whether the bile acids suppress osteoclast differentiation from the precursor RAW 264.7 cells. Pg-LPS did not induce RAW 264.7 cells to OLCs, unlike the other study to do with *E. coli* LPS<sup>25</sup>. Meanwhile, the cells were differentiated to OLCs under RANKL stimulation. However, the inhibitory effect of bile salts to osteoclastogenesis is weaker than to inflammation. The only sodium glycocholate at 1,000  $\mu\text{M}$  remarkably inhibited osteoclast differentiation, and sodium taurodeoxycholate and sodium taurocholate did not.

In the other study, sodium glycocholate inhibited the expression of a transcription factor NF- $\kappa\text{B}$  related to IL-2 as well as the production of TNF- $\alpha$  in the immune cells stimulated by *E. coli* LPS<sup>18</sup>. Since NF- $\kappa\text{B}$  is expressed in the canonical pathway by TNF- $\alpha$  or RANKL, contributing to induce the osteoclast precursors to osteoclast, the osteoclast differentiation is also suppressed as an NF- $\kappa\text{B}$  expression is inhibited. Therefore, the inhibition of osteoclast differentiation by sodium glycocholate presented in this study, might be attributed to the suppression of RANKL-derived NF- $\kappa\text{B}$  pathway by the bile acids. Meanwhile, the osteoclast differentiation was inhibited at a relatively high concentration in the study, which also corresponds the result that the activation of NF- $\kappa\text{B}$  decreased to 25-75% of the control group at 1,000-2,000  $\mu\text{M}$  of sodium glycocholate<sup>18</sup>. Thus, the required concentration of glycocholate for anti-osteoclastogenesis is thought to be higher than that for anti-inflammatory effect. On the other hand, taurodeoxycholate activated NF- $\kappa\text{B}$  and inhibited subsequent TNF- $\alpha$ -induced apoptosis in intestinal epithelial cells<sup>23</sup> and induced the increase of IL-2 in another study<sup>19</sup>. Therefore, it could be estimated that taurodeoxycholate did not affect to inhibit osteoclast differentiation because it did not interrupt the activation of RANKL-induced NF- $\kappa\text{B}$  as opposed to glycocholate. Also, it is possible that taurocholate has a similar mechanism to taurodeoxycholate.

As comprehensively considering cytotoxicity, anti-inflammatory effect, and inhibitory effect on osteoclastogenesis, glycocholate of three bile acids is regarded to be the most effective. However, the concentration of it needed to present the significant effect was relatively high as about 10-1,000  $\mu\text{M}$ , although it had no cytotoxic effect at that concentration, compared to that the effective concentrations of test compounds

were about 1-100  $\mu$ M in other studies for RAW 264.7 differentiation<sup>25,26</sup>. Thus, the effectiveness of glycocholate to TNF- $\alpha$  and osteoclast differentiation except IL-6 were not higher than other substances. However, the further studies are needed for glycocholate to reassure and determine the effective and proper concentration, and to elucidate the mechanisms in details for the inhibitory effect on inflammation and osteoclastogenesis related to an NF- $\kappa$ B pathway or other cell signaling. Although other two bile acids with a taurine group had a partial impact on the regulation of inflammation, the studies for them are also needed. However, comprehensively, these bile acids presented inhibitory effect on inflammation and related metabolism induced periodontal pathogens as well as other tissues or systems. The inflammation is common mechanism linking periodontitis and many systemic diseases. Application of bile acids could target inflammation locally periodontally and systemically. Bile acids derivatives or extracts used for other purposes could be used in periodontal tissue.

There are several limitations of the study. First, it is needed to set up the environment more similar to periodontal tissue and periodontitis. To test the effect of substances, several studies used periodontal related cell sources such as human gingival fibroblast<sup>2</sup> or mouse bone marrow macrophages (BMMs)<sup>4,27</sup>. However, this study did not use periodontium-origin cell or primary cells. Instead, periodontal pathogen Pg-LPS<sup>2,26,27</sup> and myelomonocytic cell lines developing both inflammatory cells (macrophages) and osteoclast<sup>25</sup>, were used similarly to some studies. RAW 264.7 cells were also confirmed to be developed to osteoclast, although the differentiation of BMMs is definitive. The application to other periodontium-related cells or tissues is needed. Second, there were insufficient results related to anti-osteoclastogenesis and mechanistic explanations involved in inflammatory response and osteoclast differentiation. Therefore, further studies are needed to evaluate the effect of glycocholate mainly and the other bile salts on the inflammatory osteolytic activities in periodontal environments in detail. Particularly, the interest and further studies whether they have different mechanisms with RANKL, NF- $\kappa$ B, and other osteoclastogenesis-related signaling pathway are important since these bile acids presented the contrary effects on the differentiation to osteoclast. In addition, based on the results in vitro study, the further studies including in vivo or other conditions are needed.

## Conclusions

This study was the first step to evaluate the potential of bile acids for the prevention or intervention of periodontal inflam-

mation and bone resorption as well as its potential as a universal immuno-regulator. This study was to present that bile acids had inhibitory effects to the inflammation, which is common mechanism between systemic diseases and periodontal diseases. In conclusion, three bile acids inhibited *P. gingivalis* LPS-induced inflammatory responses and glycocholate partially suppressed RANKL-mediated osteoclastogenesis in RAW 264.7 cells.

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