치의학석사 학위논문

담즙산염이 *Porphyromonas gingivalis* 리포다당질에 의해 유도된 염증반응에 미치는 효과

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

2019년 2월

서울대학교 대학원
치의학대학원
박 지 아

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by

Ji-A Park

Advisor:

Prof. Jin, Bo-Hyoung, DDS, MSD, PhD

A Thesis Submitted in Partial Fulfillment of the Requirements for the Doctor of Dental Surgery

February, 2019

School of Dentistry

Seoul National University

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박 지 아

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2018년 12월

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November, 2019

School of Dentistry

Seoul National University

Doctoral Committee:

Professor _____,Chairman

Professor _____,Vice chairman

Professor _____

Abstract

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Seoul National University
(Directed by Prof. Jin Bo-Hyoung, DDS, MSD, PhD)

Introduction

Periodontitis is a chronic inflammatory disease leading to the destruction of periodontal tissue including alveolar bone loss by immuno-inflammatory response between the host immune system and periodontal pathogens. Chronic inflammation can be described as a shared mechanism which links periodontal disease and various systemic diseases. Bile salts and its derivatives such as sodium glycocholate and sodium taurodeoxycholate are noted as the substances to regulate immune response and could suppress excessive inflammation and tissue destruction universally. However, there is no study to assess their potentials to periodontal inflammation. Therefore, this study aimed to evaluate the inhibitory effect of bile salts on the inflammatory response and osteoclastogenesis in macrophage cell-line RAW 264.7 stimulated by periodontal pathogen *Porphyromonas gingivalis*

lipopolysaccharide (LPS) and a receptor activator of nuclear factor-κB ligand (RANKL).

Materials and Methods

The myelomonocytic RAW 264.7 cells were activated by *P. gingivalis*-LPS to induce inflammatory responses, three bile salts including sodium taurodeoxycholate, sodium taurocholate, and sodium glycocholate were treated at different concentrations. The cytotoxicity effect of bile salts was assessed by measuring cell viability with MTT-assay. To evaluate their inhibitory effect to the inflammatory responses, we applied the enzyme-linked immunosorbent assay method to measure the induction levels of the pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α at 12 hours after treatment of the reagents. In addition, after the cells were stimulated by macrophage colony-stimulating factor and RANKL to promote osteoclastogenesis and treated with three bile salts, it was examined whether they suppress osteoclast differentiation by tartrate-resistant acid phosphatase-staining.

Results

In cell viability test, sodium taurodeoxycholate and sodium taurocholate had no cytotoxic effect on RAW 264.7 cells at the concentration of equal or less than 200 μM, while sodium glycocholate was non-cytotoxic up to the maximal concentration of 4,000 μM. In the evaluation for their inhibitory effect to inflammatory response, the production levels of pro-inflammatory cytokines IL-6 and TNF-α decreased according to the increasing

of concentration of three bile salts in a dose-dependent manner. The expression of IL-6 reduced remarkably in the treatment of sodium taurodeoxycholate and sodium glycocholate ($p < 0.001$), while the production of TNF-α decreased with the treatment of sodium glycocholate and the significance was relatively weak ($p < 0.05$). In the evaluation for the effect of bile salts to osteoclastogenesis, only sodium glycocholate at the concentration of 1,000 μM suppressed osteoclast differentiation of RAW 264.7 cells ($p < 0.001$), while sodium taurodeoxycholate and sodium taurocholate had no inhibitory effect to osteoclastogenesis.

Conclusions

Three bile salts sodium taurodeoxycholate, sodium taurocholate, and sodium glycocholate inhibited *P. gingivalis* LPS-induced inflammatory response, and sodium glycocholate at high concentration suppressed RANKL-mediated osteoclastogenesis in RAW 264.7 cells.

Keywords: bile salt, periodontitis, inflammation, osteoclast differentiation, *Porphyromonas gingivalis*, glycocholate, taurocholate, taurodeoxycholate

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Abbreviations

ANOVA	Analysis of variance
BMMs	Bone marrow-derived macrophages
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
GPCR19	G-protein coupled receptor 19
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OLCs	Osteoclast-like cells
P. gingivalis	*Porphyromonas gingivalis*
Pg-LPS	*Porphyromonas gingivalis* 33277 LPS
RANKL	Receptor activator of NF-κB ligand
TNF-α	Tumor necrosis factor-alpha
TRAP	Tartrate-resistant acid phosphatase

Ⅰ. Introduction

1. Background

1.1. The pathogenesis of periodontitis

Periodontitis is a chronic inflammatory disease leading to the destruction of periodontal tissue including alveolar bone loss by immuno-inflammatory response between the host immune system and periodontal pathogens. Its prevalence is high as a range of 10-60% globally (Xiong et al., 2006), and severe periodontitis was identified as the sixth most prevalent disease worldwide (10.8%) (Kassebaum et al., 2014) and as a major cause of tooth loss in adults.

As a multifactorial disease, periodontitis is involved with local oral microorganisms, environmental conditions, and host factors. Depending on host-specific susceptibility to microbial and environmental factors, the host-immune system often responses excessively to aggregate inflammatory cells and increase the expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, proteolytic enzymes, and bone metabolism-related cytokines, finally resulting in destruction of periodontal tissue.

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* presents a strong pathogenic effect with a variety of adhesion molecules and proteolytic enzymes such as gingipains.

In addition, its distinct LPS evades host recognition (Martin et al., 2001) and secondly induces the secretion of pro-inflammatory cytokines in periodontal tissue, thereby activating a receptor activator of nuclear factor-κB ligand (RANKL) expression and osteoclast, resulting in alveolar bone destruction (Yamamoto et al., 2006; Kong et al., 2015; Lee et al., 2018).

1.2. Periodontal diseases, systemic diseases and chronic inflammation as their link

Meanwhile, the periodontal pathogens, its endotoxin, and local inflammatory mediators in periodontal tissue were found to be able to induce systemic inflammations through blood vessels developed in periodontal tissue. (Ebersole et al., 2003; Seymour et al., 2007; Andrukhov et al., 2011) The level of an important systemic inflammatory marker such as C-reactive protein and pro-inflammatory cytokines were increased in chronic periodontal infection, and they were also high in patients with systemic conditions such as obesity, type-2 diabetes, metabolic syndrome, and cardiovascular disease (Ioannidou et al., 2011; Esser et al., 2014).

Accordingly assuming that the periodontal inflammation may interact with the systemic inflammatory process to cause or exacerbate systemic diseases, numerous studies have been conducted on the association between oral health and various systemic diseases (Sabharwala et al., 2018). Several epidemiological and clinical studies have shown that many chronic complex systemic conditions, including metabolic syndrome, cardiovascular disease, and diabetes, are associated

with periodontitis (Lopez et al., 2011; Nibali et al., 2013; Sabharwala et al., 2018). Therefore, the forceful intervention of periodontitis is important in aspects of the interaction with systemic conditions as well as the high burden of the disease.

In recent year, additionally, peri-implant diseases with similar characteristics to periodontal diseases also have increased as implant replacements for lost teeth have become common. The prevalence of peri-implantitis and peri-implant mucositis were reported as high as about 20% and 47%, respectively (Lee et al., 2017). The periodontopathogens closely related to periodontitis such as *P. gingivalis* and *Treponema denticola* were also found at high levels in inflammatory peri-implant sites. (Sanz-Martin et al., 2017) and the association between peri-implantitis and systemic conditions such as metabolic syndrome was reported recently (Papi et al., 2018).

Comprehensively, chronic inflammation can be described as a shared mechanism of linking periodontal diseases including periodontitis and peri-implantitis, and various systemic diseases. It has become more important as a critical factor in the pathogenesis of many common systemic diseases, and therefore, the control of the inflammatory process is an essential target for the management of both periodontal and systemic diseases as inhibiting the negative interactions among them.

1.3. Bile salts as a regulator for inflammatory responses

These days, many studies have been conducted actively on therapeutic compounds or compositions capable of regulating abnormal immuno-inflammatory process and tissue destructive

metabolism, related to the systemic inflammatory diseases. Especially, there are several types of research on the new role of substances originated from natural sources and well-known bio-compounds as an inflammatory regulator (Ma et al., 2015; Kong et al., 2015) These universally applied substances to suppress inflammatory process and related tissue destructions are also likely to be effective to periodontal inflammation and subsequent alveolar bone loss.

Bile salts have been noted as the potentials as an immuno-regulator to suppress excessive inflammation (used as general purpose). Studies have been carried out to evaluate the novel effects of them as a regulator for various inflammatory diseases and conditions, beyond the role of a surfactant for lipid degradation (Seong et al., 2007, 2013, and 2014; Yang at al., 2016).

Sodium glycocholate, as one of the bile salts, presented an inhibitory effect on inflammatory activation induced by *E. coli* LPS in a study to find anti-inflammatory agents. It inhibited the activations of dendritic cells by LPS and subsequent T cells and also reduced the expressions of pro-inflammatory cytokines such as TNF-α and IL-12p40 in murine sepsis model (Seong et al., 2007). Another bile salt taurocholate also decreased the levels of IL-1β, interferon (IFN)-ɣ and TNF-α in colon tissues and alleviated inflammatory signs in the study for its anti-inflammatory effect on ulcerative colitis (Yang at al., 2016).

Also, sodium taurodeoxycholate, which is an agonist of the G-protein coupled receptor 19 (GPCR19) for a membrane receptor that recognizes bile acids, was examined as a therapeutic agent to prevent sepsis and atopic dermatitis. It suppressed septic

response triggered with LPS by amplifying immuno-regulatory cells and alleviated abnormal inflammatory reactions on cutaneous tissues (Seong et al., 2013 & 2014). Another study also reported that taurodeoxycholate promoted the proliferation of intestinal cells and inhibited the apoptosis induced by the activation of NF-κB in a dose-dependent manner (Toledoe et al., 2004).

2. Purpose

The bile salts could also be effective to control the inflammatory process in periodontal tissue as presented the anti-inflammatory effects in the several tissues and disease models universally. They could affect the local and general inflammatory process simultaneously as considering that periodontal and systemic disease share the mechanism of chronic inflammation. However, there is no study to evaluate whether bile salts inhibited periodontal inflammation and accompanied tissue destruction such as alveolar bone loss. Therefore, it is necessary to investigate the potentiality of these universal inflammatory regulators exert on periodontitis. This study aimed to evaluate the inhibitory effect of bile salts on the inflammatory response and osteoclastogenesis in macrophage cell line RAW 264.7 stimulated by periodontopathogens *P. gingivalis*-LPS and RANKL.

Ⅱ. Materials and Methods

1. Bile salts and reagents

Three bile salts were selected as the experimental substances; 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 macrophages cell-line RAW 264.7 cells (ATCC® TIB-71TM; American Type Culture Collection, Manassas, VA, USA) were used. This myelomonocytic cell line was selected as an experimental one since macrophages are immune cells regulating inflammatory response and derived from monocytes, which can differentiate into osteoclasts depending on the surrounding regulatory factors such as M-CSF, RANKL, and the 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), and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma) in 100 mm culture dishes (Corning, Corning,

NY, USA), and incubated 37°C and 5% CO_2 under 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, known as the most critical periodontopathogen 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 salts 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 more for different hours according to the analyses.

3. MTT assay for cell viability test

Cell viability or cell cytotoxicity was assessed by MTT-assay. RAW 264.7 cells were seeded at a density of 3×10^3 cells/well onto 96-well plates and incubated in a 37°C and 5% CO_2 for 24 hours. The wells were treated then with either only culture medium 100 μl (control) or three bile salts at different concentrations with or without Pg-LPS (1 μg/ml); sodium taurodeoxycholate and 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 MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Alfa Aesar)

solution was added in each wells. 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. Each point is repeatedly measured (n=6).

4. ELISA for cytokine detection

RAW 264.7 cells were cultured at a density of 3×10^4 cells/well in 6-well plates and incubated in a 37°C and 5% CO_2 for 24 hours. The wells treated then with either culture medium alone (control) and three bile salts of serial concentrations under the stimulation of Pg-LPS (1 μg/ml). According to the results of cytotoxicity test, sodium taurodeoxycholate and sodium taurocholate were treated at a concentration in the range of 0-100 μM (0, 1, 10, and 100 μM) and sodium glycocholate with a concentration in the range of 0-1,000 μM (0, 1, 10, 100, and 1,000 μM) in the limited concentration without cell toxicity effect. Culture supernatants were collected after incubating for 12 hours (the treatment time was determined by the preliminary experiments with several time points), and stored at -80℃.

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), PBS with 0.05% Tween 20 (Intron Biotechnology, Seongnam, Korea) for wash buffer, 1M phosphoric acid (Alfa Aesar) for stop solution, and high-affinity 96-well plates (Corning) were used for cytokine

analyses in accordance with the manufacturer's instructions. After reagent treatments, the optical density of the plates was measured at 450 nm. Each point is repeatedly measured (n=3).

5. TRAP staining for osteoclastogenesis detection

2×10^3 cells/well of RAW 264.7 cells were seeded in 96-well plates and treated with Pg-LPS (1 μg/ml) or RANKL (100 ng/mL) combined with M-CSF (30 ng/mL) in the absence or presence of three bile salts and 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. Thus, 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 ≥ 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). Each point is repeatedly measured (n=4).

6. Statistical analysis

All experiments were performed at least in duplicate or

triplicates. Results were presented as the mean and standard deviation (S.D). One-way analysis of variance (ANOVA) followed by Tukey test were used in the statistical analyses and p-values < 0.05 were considered statistically significant. All statistical analyses were performed by Graphpad Prism version 5.01 software (Graphpad software Inc., Graphpad San Diego, CA, USA).

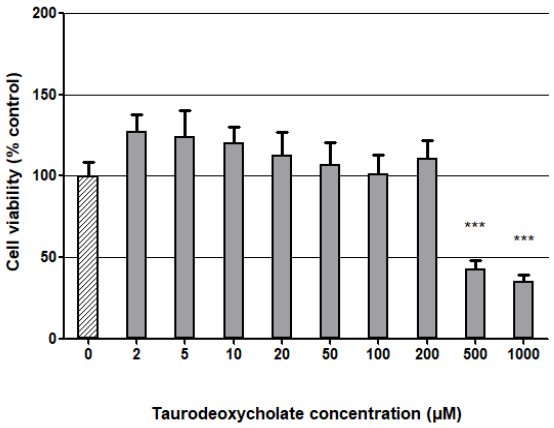
Ⅲ. Results

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

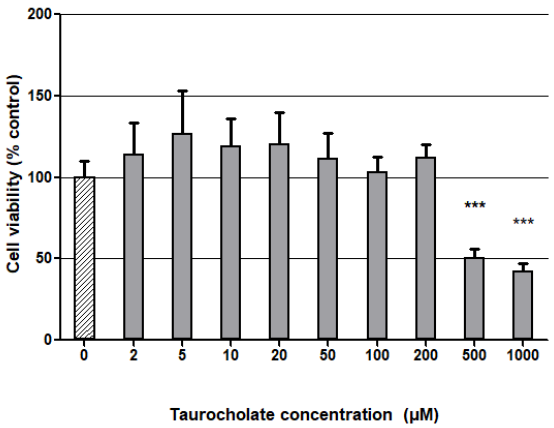
RAW 264.7 cells were treated with culture medium alone or in combination with bile salts, and cell viability was measured after 24 hours by MTT-assay. In results, the cell viabilities of three bile salts-treated groups were not significantly different compared to that of the control group at most concentrations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

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 μM. However, both groups presented cytotoxicity to the cells 500 and 1,000 μM ($p < 0.001$) (Figure 1A, B). Meanwhile, the sodium glycocholate presented nontoxic effect at all concentrations to 4,000 μM, a maximum concentration in the experiment (Figure 1C). It also corresponds to the other in vitro study that reported sodium glycocholate at 1 mg/ml or less (about 2,000 μM) was appropriate (Seong et al., 2007).

A


Cell viability (% control)
200
150
100
50
0
***
***
0
2
5
10
20
50
100
200
500
1000
Taurodeoxycholate concentration (μM)

B


Cell viability (% control)
200
150
100
50
0
***
***
0
2
5
10
20
50
100
200
500
1000
Taurocholate concentration (μM)

C

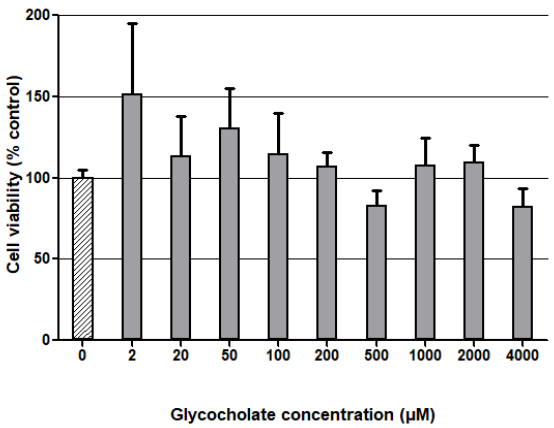

Cell viability (% control)
200
150
100
50
0
0
2
20
50
100
200
500
1000
2000
4000
Glycocholate concentration (μM)

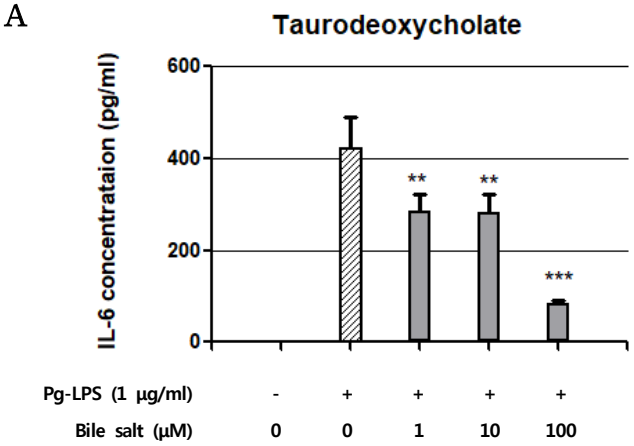
Figure 1. Effect of bile salts on the viability of RAW 264.7 cells.

RAW 264.7 cells (3×10^3cells/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 μM. 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 μM ($p < 0.001$). (C) Sodium glycocholate at the range of 0-4,000 μM and presented no cytotoxic effect at all concentrations up to a maximal 4,000 μM. Results were expressed as the relative cell viabilities (%) compared to the control group (no bile salt; 0 μM) as a reference. Bar and error bar present the mean ± S.D. of % cell viability relative to the control. *** $p < 0.001$ significantly different from the control. Each point is repeatedly measured (n=6).

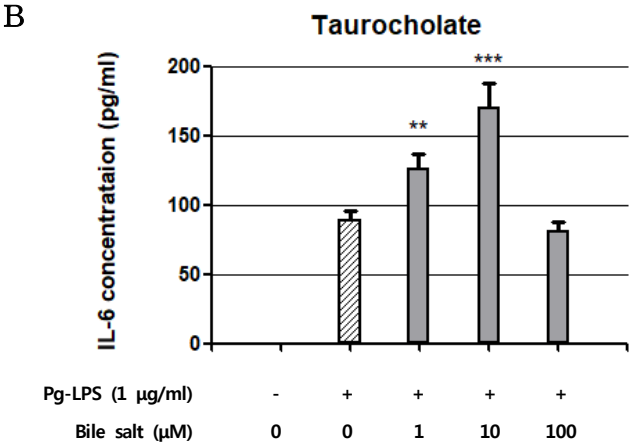
2. Three bile salts inhibited the production of pro-inflammatory cytokines IL-6 and TNF-α in Pg-LPS-stimulated RAW 264.7 cells

To assess the effect of three bile salts to inflammatory responses, the induction levels of the pro-inflammatory cytokines IL-6 and TNF-α at 12 hours after treatment of the reagents were measured by ELISA. The production of IL-6 and TNF-α increased significantly in the cell supernatants which were stimulated under Pg-LPS alone compared to the control ones, while the expression of both cytokines tended to be suppressed with the increasing of concentration of three bile salts in a dose-dependent manner (Figure 2A-F). Notably, the production of IL-6 was strongly inhibited at concentrations of 100 μM and 10 μM or higher in sodium taurodeoxycholate and sodium glycocholate, each ($p < 0.001$) (Figure 2A-C). The induction of TNF-α 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-α were significantly lowered only in the treatment of 1,000 μM sodium glycocholate ($p < 0.05$) (Figure 2D-F).

A


Taurodeoxycholate
IL-6 concentrataion (pg/ml)
600
400
200
0
**
**
***
Pg-LPS (1 μg/ml) - + + + +
Bile salt (μM) 0 0 1 10 100

B


Taurocholate
IL-6 concentrataion (pg/ml)
200
150
100
50
0
**
***
Pg-LPS (1 μg/ml) - + + + +
Bile salt (μM) 0 0 1 10 100

C

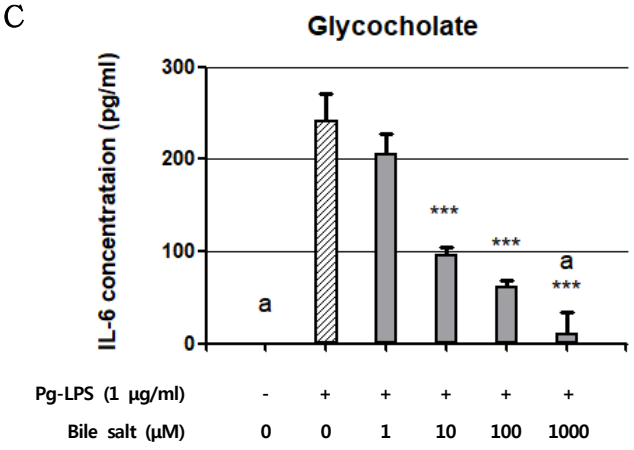
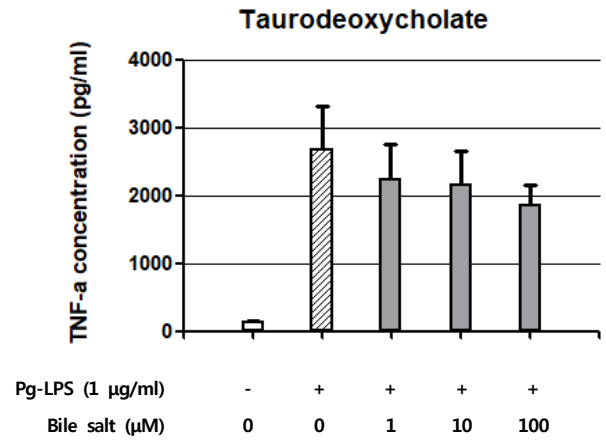

Glycocholate
IL-6 concentrataion (pg/ml)
300
200
100
0
a
***
***
a
***
Pg-LPS (1 μg/ml) - + + + + +
Bile salt (μM) 0 0 1 10 100 1000

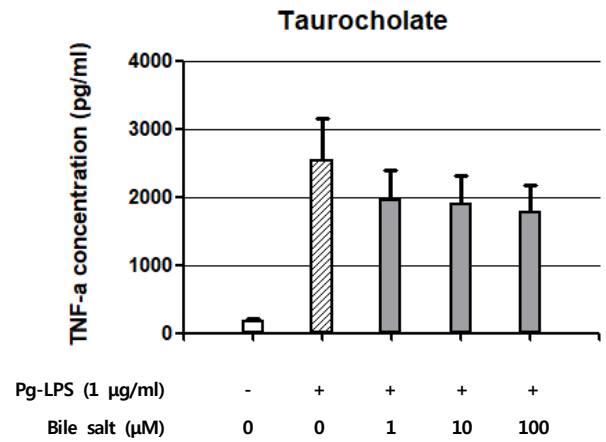
Figure 2. Effects of three bile salts on expression of pro-inflammatory cytokines in Pg-LPS-stimulated RAW 264.7 cells.

RAW 264.7 cells were cultured at a density of 3×10^4 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 μM or 0-1,000 μM under the stimulation of Pg-LPS (1 μg/ml) for 12 hours. The levels of pro-inflammatory cytokines IL-6 and TNF-α in the culture supernatants were measured by ELISA. The production of IL-6 (A-C) and TNF-α (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 μM and 10 μM in sodium taurodeoxycholate and sodium glycocholate treatment, each ($p < 0.001$). Results (bar and error bar) present the mean ± S.D. of cytokine levels (pg/ml). ** $p < 0.01$ and *** $p < 0.001$ significantly different from only Pg-LPS group. 'a' means no significant differences with the control (no Pg-LPS) group.

D


Taurodeoxycholate
TNF-a concentration (pg/ml)
4000
3000
2000
1000
0
Pg-LPS (1 μg/ml) - + + + +
Bile salt (μM) 0 0 1 10 100

E


Taurocholate
TNF-a concentration (pg/ml)
4000
3000
2000
1000
0
Pg-LPS (1 μg/ml) - + + + +
Bile salt (μM) 0 0 1 10 100

F

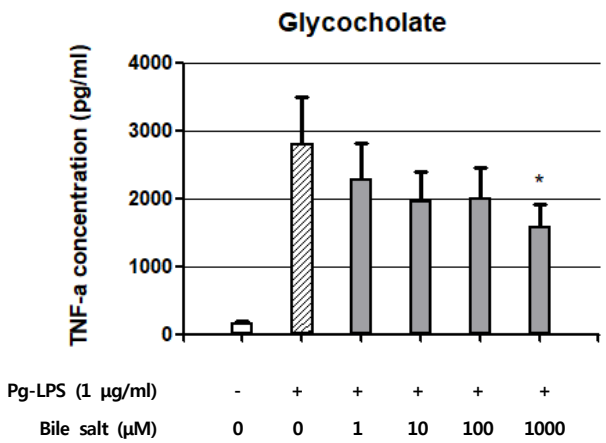

Glycocholate
TNF-a concentration (pg/ml)
4000
3000
2000
1000
0
*
Pg-LPS (1 μg/ml) - + + + + +
Bile salt (μM) 0 0 1 10 100 1000

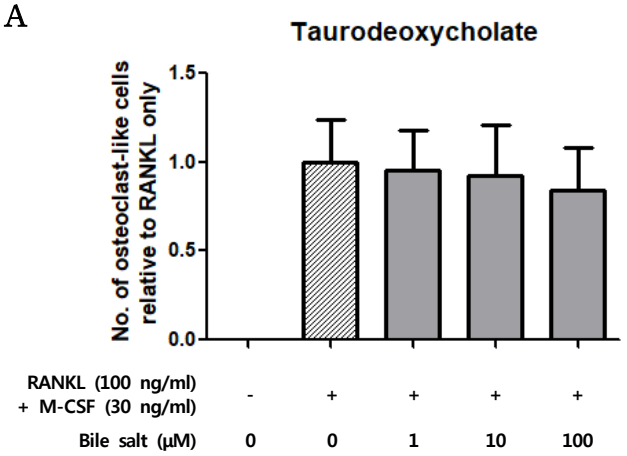
Figure 2 (continued).

(D-F) The production of TNF-α was suppressed as the concentration of three bile salts increased although the significance was relatively weak than IL-6. The levels of TNF-α were significantly lowered only in the treatment of sodium glycocholate at 1,000 μM ($p < 0.05$). Results (bar and error bar) present the mean ± S.D. of cytokine levels (pg/ml). * $p < 0.05$ significantly different from only 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-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay.

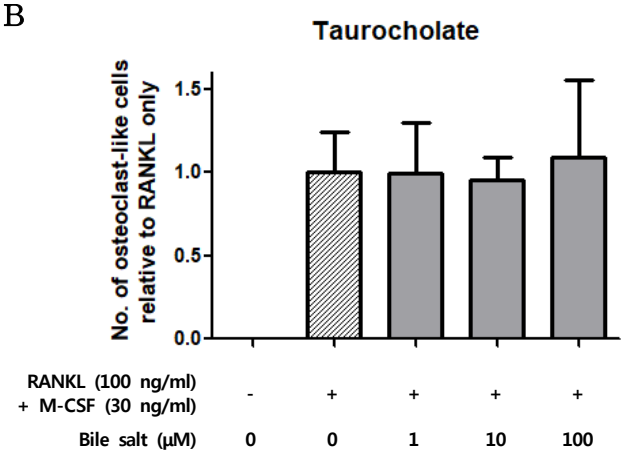
3. Sodium glycocholate at the highest concentration remarkably suppressed osteoclast differentiation of RAW 264.7 cells

To evaluate the effect of bile salts to osteoclastogenesis induced by Pg-LPS or RANKL with 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, the precursor of osteoclast RAW 264.7 cells stimulated by Pg-LPS was 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 treated bile salts with RANKL and M-CSF were no significant difference compared to those of the only RANKL and M-CSF group, except the conditions with the treatment of sodium glycocholate (Figure 3A-C). The sodium glycocholate at the highest concentration of 1,000 μM remarkably suppressed osteoclast differentiation of RAW 264.7 cells ($p < 0.001$) (Figure 3C, D), while sodium taurodeoxycholate and sodium taurocholate were no significant effect on the inhibition of osteoclastogenesis (Figure 3A, B).

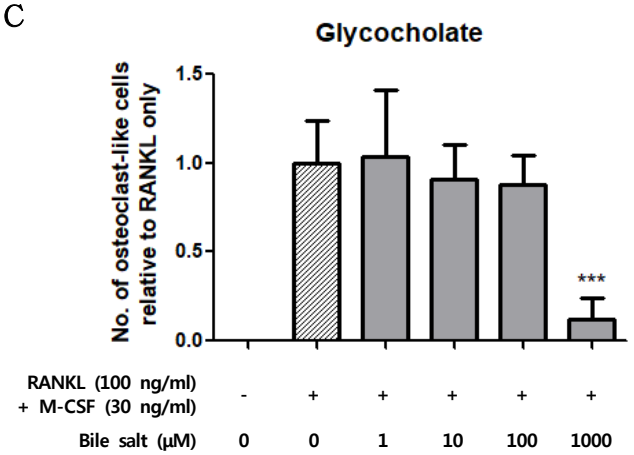
A


Taurodeoxycholate
No. of osteoclast-like cells relative to RANKL only
1.5
1.0
0.5
0.0
RANKL (100 ng/ml) + M-CSF (30 ng/ml)
-
+
+
+
+
Bile salt (μM)
0
0
1
10
100

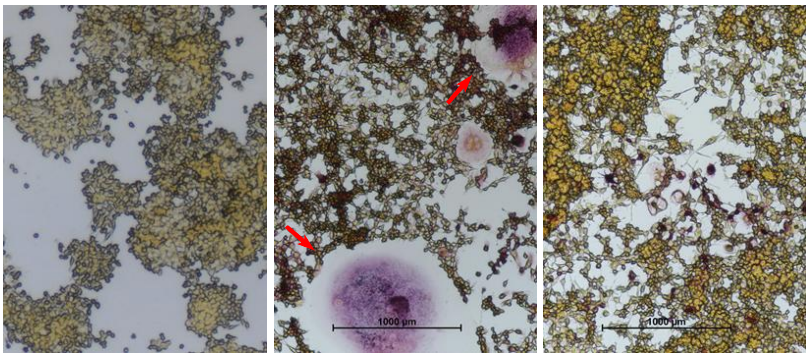
B


Taurocholate
No. of osteoclast-like cells relative to RANKL only
1.5
1.0
0.5
0.0
RANKL (100 ng/ml) + M-CSF (30 ng/ml)
-
+
+
+
+
Bile salt (μM)
0
0
1
10
100

C


Glycocholate
No. of osteoclast-like cells relative to RANKL only
1.5
1.0
0.5
0.0
***
RANKL (100 ng/ml) + M-CSF (30 ng/ml)
-
+
+
+
+
+
Bile salt (μM)
0
0
1
10
100
1000

D



	Control	RANKL only	Glycocholate
RANKL (100 ng/ml) + M-CSF (30 ng/ml)	-	+	+
Bile salt (μM)	0	0	1000

Figure 3. Effects of three bile salts on osteoclast differentiation of RAW 264.7 cells stimulated by RANKL.

(A-C) RAW 264.7 cells (2×10^3cells/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 μM. After 4 days, the cells were fixed, and TRAP staining was carried out. TRAP-positive multinucleated cells (with ≥ 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

μM (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 glycocholate treatment group. The RANKL only group presented many multinucleated giant cell as the cells indicated red arrows, while the cells with glycocholate 1,000 μM are 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 ± 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-κB ligand; M-CSF, macrophage colony-stimulating factor; TRAP, tartrate-resistant acid phosphatase; OLCs, osteoclast-like cells.

Ⅳ. Discussion

This study aimed to evaluate the inhibitory effect of bile salts on the inflammatory response and osteoclastogenesis in myelomonocytic cells RAW 264.7 stimulated by periodontopathogen *P. gingivalis*-LPS and RANKL. The study assessed the effect of bile salts on the cells related to both immune and bone metabolisms considering periodontal inflammation. Although several studies evaluated the effect of bile salts on other immune cells and inflammatory disease models such as atopic dermatitis, ulcerative colitis, and sepsis, there is no study to examine the effect on periodontitis or osteoclast differentiation. The study evaluated first osteoclast differentiation because the process of bone loss accompanied by an inflammatory response is essential 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 or less than 200 μM, while sodium glycocholate did at the maximal concentration of 4,000 μM. Thus, the sodium glycocholate can be applied in a broader range of concentration. The results correspond to the previous study that reported the sodium glycocholate had the anti-inflammatory effect without cytotoxicity within a range of 0.01-1 mg/ml (about 20-2,000 μM) on other immune cells (Seong et al., 2007). In addition, another study suggested the most appropriate concentration of sodium taurodeoxycholate applied to bone marrow-derived immune cells

was 0.05 mg/ml (about 100 μM) (Seong et al., 2007). On the other hand, taurodeoxycholate at 500-1,000 μM had no toxic effect to intestinal epithelial cell lines and rather promoted their proliferation in the other study (Toledoe et al., 2004). It may be due to differences in the types of cells such as immune cells and epithelial cells and their cell signal mechanisms.

To assess the inhibitory effect of three bile salts to inflammatory responses, the production levels of the pro-inflammatory cytokines IL-6 as well as TNF-α were measured in the conditions combined with Pg-LPS and three bile salts. The expression of IL-6 decreased according to increasing of concentration of bile salt, particularly did significantly in sodium taurodeoxycholate and sodium glycocholate. This study first confirmed these bile salts had the inhibitory effect on IL-6 in the immune cells stimulated by a periodontopathogen Pg-LPS. The treatment of bile salts also inhibited the expression of TNF-α in a dose-dependent manner. Several studies have reported these bile salts had the anti-inflammatory effect of reducing TNF-α and IL-1β in other tissues including inflamed colon tissue, cutaneous tissue as well as immune cells under another type of LPS stimulation (Seong et al., 2007, 2014; Yang et al., 2016). The study presented a similar anti-inflammatory effect on myelomonocytic immune cells activated by Pg-LPS. However, the effect was stronger to IL-6 than TNF-α. The inhibition levels of TNF-α correspond the study where the treatment of glycocholate at 1,000 μM decreased the expression levels of TNF-α to about 50 % compared to the control in primary macrophages (Seong et al., 2007). Meanwhile, sodium taurodeoxycholate at 100 μM, which was suggested as the optimal concentration in another study, had

a weak effect to TNF-α in this study. Considering the concentration with non-cytotoxic effect, sodium taurodeoxycholate and especially sodium glycocholate are effective to the inhibition of inflammatory mediators.

This study also first examined whether the bile salts 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 (Kats et al., 2016). 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 the highest concentration (1,000 μM) 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-κB which is involved to the production of IL-2 as well as reduced the production of TNF-α in the immune cells stimulated by *E.coli* LPS (Seong et al., 2007). Since NF-κB is expressed in the canonical pathway by TNF-α or RANKL, contributing to induce the osteoclast precursors to osteoclast, the osteoclast differentiation is also suppressed as an NF-κB 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-κB pathway by the bile salt. Meanwhile, the osteoclast differentiation was inhibited at a relatively high concentration in the study, which also corresponds the result that the activation of NF-κB decreased to 25-75 % of the control group at 1,000-2,000 μM of sodium glycocholate (Seong et al.,

2007). Thus, the required concentration of sodium glycocholate for anti-osteoclastogenesis is thought to be higher than that for anti-inflammatory effect.

On the other hand, taurodeoxycholate activated NF-κB and inhibited subsequent TNF-α-induced apoptosis in intestinal epithelial cells (Toledoe et al., 2004) and induced the increase of IL-2 in another study (Seong et al., 2014). Therefore, it could be estimated that the treatment of sodium taurodeoxycholate did not affect to inhibit osteoclast differentiation because it did not interrupt the activation of RANKL-induced NF-κB as opposed to sodium glycocholate. Though the mechanism of taurocholate to affect osteoclast differentiation has not been elucidated yet, there is the possibility that it is similar to the mechanistic explanation for taurodeoxycholate. Further confirmation of how taurocholate affects the NF-κB pathway will be needed.

As consider the complex cytotoxicity, anti-inflammatory effect, and inhibitory effect on osteoclastogenesis, sodium glycocholate of three bile salts is regarded to be the most effective. However, the concentration of it needed to present the significant effect was relatively high about 10-1,000 μM although it had no cytotoxic effect at that concentration, compared to that the effective concentrations of test compounds were most about 1-100 μMs in other studies for RAW 264.7 differentiation (Bian et al., 2016; Kats et al., 2016). Thus, the effectiveness (or potency) of sodium glycocholate to TNF-α and osteoclast differentiation except IL-6 were not higher than other substances. However, the further studies are needed for sodium 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-κB pathway or another cell signaling. Although other two bile salt with a taurine group had a partial impact on the regulation of inflammation, the studies for them are also needed.

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 (Kong et al., 2015) or mouse bone marrow macrophages (BMMs) (Lee, 2017; Lee et al., 2018). However, this study did not use periodontium-origin cell or primary cells. Instead, periodontal pathogen Pg-LPS (Bian et al., 2016; Kong et al., 2015; Lee et al., 2018) and myelomonocytic cell lines which can develop both inflammatory cells of macrophages and osteoclast (Kats et al., 2016), were used similarly to other studies. RAW 264.7 cells are 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, as above mentioned, there are 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 main glycocholate and the other bile salts on the inflammatory osteoclastic activities in periodontal environments in detail. Particularly, the interest whether they have different mechanisms about RANKL, NF-κB, and other osteoclastogenesis-related signaling pathway is important since these bile salts presented the contrary effects on the differentiation to osteoclast. Also, further research would make the results more consistent and

clear as increasing of experimental precision. In addition, based on the results *in vitro* study, the further studies including *in vivo* or other conditions are needed.

Ⅴ. Conclusion

This study was the first step to evaluate the bile salts as the potential therapeutic compositions for the prevention and intervention of periodontal inflammation as well as the potential as a universal immuno-regulator. The study was to present results related bile salts considering the interaction between systemic diseases and periodontal diseases mediated shared chronic inflammation. In conclusion, three bile salts inhibited *P. gingivalis* LPS-induced inflammatory response and sodium glycocholate at high concentration suppressed RANKL-mediated osteoclastogenesis in RAW 264.7 cells.

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국문초록

담즙산염이 *Porphyromonas gingivalis* 리포다당질에 의해 유도된 염증반응에 미치는 효과

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치의학대학원
(지도교수: 진보형)
박 지 아

1. 목 적

대표적인 구강상병인 치주질환은 만성염증질환으로, 치주병원균과 숙주면역체계 등의 상호작용으로 발생한 지속적인 면역염증반응이 치조골 등의 치아주위조직의 파괴를 유발하는 질환이다. 치주염은 많은 전신질환과 함께 만성염증의 기전을 공유하여, 염증조절은 질병 관리의 중요한 목표이다. 한편, 담즙산염은 면역염증과정을 조절할 수 있는 물질로서 가능성을 주목받고 있는데, 패혈증이나 궤양성 대장염, 아토피성 피부염 등에 대한 몇몇 담즙산염의 효과를 평가하는 여러 실험연구에서 면역세포활성 조절 및 염증반응 억제 등에 효과를 보이는 것으로 나타났다. 그러나 치주질환에서 담즙산염의 효과를 평가하는 연구가 부재한 실정이다. 그래서 이번 연구는 주요 치주병원균인 *Porphyromonas gingivalis*의 리포다당질(lipopolysaccharide, LPS)과 RANKL을 이용하여, 담즙산염이 치주질환의 염증과정 및 골대사 등의 조직파괴과정에 억제효과를 보일

수 있을지에 대한 가능성을 평가하고자 한다.

2. 방 법

대식세포 세포주인 RAW 264.7에 치주질환의 주요 병원균으로 알려진 *P. gingivalis* LPS를 접종하여 면역염증반응을 유도하고, 3종의 담즙산염 타우로데옥시콜산염(sodium taurodeoxycholate) 및 타우로콜산염(sodium taurocholate), 글리코콜산염(sodium glycocholate) 각각을 농도별로 처리하여 효과를 확인하였다. MTT 측정법으로 세포생존 정도를 측정하여 각 물질의 세포독성효과를 확인하고, 효소결합면역흡착측정법으로 대표적인 염증성 사이토카인 TNF-α 및 IL-6의 생성 정도를 측정하여 각 물질의 염증반응에 대한 억제효과를 확인하였다. 또한, RAW 264.7에 *P. gingivalis* LPS 및 M-CSF, RANKL을 처리하여 파골세포로의 분화를 유도하고, 3종의 물질을 각각 농도별로 처리한 후, TRAP 염색법으로 파골세포양세포를 확인하여 파골세포로의 분화 정도를 확인하였다.

3. 결 과

세포독성평가에서 타우로데옥시콜산염 및 타우로콜산염 처리군은 200 μM 이하의 농도에서 대조군과 세포생존 정도에 유의한 차이가 없었고, 글리코콜산염 처리군은 실험최고농도인 4,000 μM 이하의 전 농도에서 세포독성을 보이지 않았다. 염증반응평가에서 담즙산염 처리 12시간 후 염증성 사이토카인 생성은 각 물질의 처리 농도가 높아질수록 억제되는 경향이었다. 특히, IL-6의 생성은 타우로데옥시콜산염 100 μM 및 글리코콜산염 10 μM 이상 농도에서 강하게 억제되었다($p < 0.001$). TNF-α 생성 또한 각 물질의 농도가 높아질수록 억제되었으나, 유의성은 상대적으로 약했다. 전반적으로 담즙산염 및 그 유도체는 유의한 세포독성을 보이지 않으며, *P. gingivalis* LPS에 의한 염증반응을 억제하는 것으로 나타났다. 담즙

산염의 파골세포 분화에의 영향은 글리코콜산염에서만 확인할 수 있었다. 글리코콜산염을 1,000 μM 처리 시 RAW 264.7의 파골세포로의 분화가 현저하게 억제되었으나 ($p < 0.001$), 타우로데옥시콜산염과 타우로콜산염은 대조군과 비교 시 파골세포 분화에 유의한 영향을 주지 않았다.

4. 결 론

타우로데옥시콜산염 및 타우로콜산염, 글리코콜산염을 비롯한 담즙산염은 *P. gingivalis* LPS로 유도된 염증반응을 억제하였고, 글리코콜산염은 높은 농도에서 RANKL에 의한 RAW 264.7 세포의 파골세포로의 분화를 억제하였다.

주요어: 담즙산염, 치주염, 염증, 파골세포분화, *Porphyromonas gingivalis*, 글리코콜산염, 타우로데옥시콜산염, 타우로콜산염
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