Thesis for the Degree of Master of Science

Chitosan oligosaccharides suppress oxidized LDL-induced foam cell formation and inflammatory response in RAW264.7 macrophages

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August, 2022

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RAW264.7 대식세포에서 키토산올리고당의 oxLDL 의 의한 거품세포 형성 및 염증 반응 억제 효능

Advisor: Prof. Jae-Young Je

By

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A dissertation

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Abbreviations

COS: Chitosan oligosaccharides

WHO: World Health Organization

LDL: Low density lipoproteins

oxLDL: oxidized low-density lipoprotein

HO-1: Heme oxygenase-1

SR: Scavenger receptors

TNF- α: tumor necrosis factor- α

IL-1 β: Interleukin 1- β

IL-6: Interleukin-6

iNOS: nitric oxide synthase

ROS: reactive oxygen species

PGE2: prostaglandin E2

COX-2: cyclooxygenase-2

apoA1: apolipoprotein A1

Nrf2: Nuclear factor erythroid 2-related factor 2

ARE: Antioxidant Response Element

ERK: Extracellular signal-regulated kinase

JNK: c-Jun NH2-terminal kinase

LOX-1: Lectin-like oxLDL receptor-1

RCT: Reverse cholesterol transport

SR: Scavenger receptors

ABC: ABC-binding cassette

FBS: Fetal bovine serum

TBS: Tris-buffered saline

$CuSO_4$: Copper sulfate

MTT: 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DMEM: Dulbecco's modified eagle's medium

EDTA: Ethylenediaminetetraacetic acid

TBA: Thiobarbituric acid

TCA: Trichloroacetic acid

TBARS: Thiobarbituric acid-reactive substances

NaOH: Sodium hydroxide

MDA: Malondialdehyde

CO: Carbon monoxide

CO_2: Carbon dioxide

DMSO: Dimethyl sulfoxide

NO: Nitric oxide

DCFH-DA: Dichloro-dihydro-fluorescein diacetate

NBD:
N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) -23,24-bisnor-5-xholen-3β-l

S.D.: Standard Deviation

MW: Molecular Weight

DP: Degree of Polymerization

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Abstract

Chitosan oligosaccharides (COS) are derived from chitosan or chitin which has been described to exhibit anti-inflammatory, antioxidant, anti-cancer properties and among others. The present of this research, COS had been used to investigate the inhibition of foam cell formation and anti-inflammatory effect in RAW264.7 macrophages stimulated with oxidized low-density lipoprotein (oxLDL). COS were found to have an inhibitory effect on foam cell formation. During ox-LDL stimulation of RAW264.7 macrophages, COS decreased cholesterol influx and SR-A1 and CD-36 protein expression levels while increasing cholesterol efflux and ABCA-1 and ABCG-1 protein expression levels. These findings imply that COS may have an inhibitory effect on foam cell formation. By reducing the production of reactive oxygen species (ROS), COS have shown promising anti-inflammatory properties by blocking the production of pro-inflammatory

cytokines like tumor necrosis factor-alpha (TNF-α), and interleukin-1 (IL-1β), and interleukin-6 (IL6). Furthermore, COS in oxLDL-stimulated RAW264.7 macrophages increase heme oxygenase 1 (HO-1) expression whereas it suppresses iNOS and COX-2 expression. Moreover, COS significantly enhances the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2). According to these findings, COS may exert their protective effect by activating the Nrf2/HO-1 pathway.

Key words: Chitosan oligosaccharides, foam cell formation, anti-inflammatory, oxLDL, RAW264.7 macrophages.

1. Introduction

1.1 Chitosan oligosaccharides (COS)

Chitosan oligosaccharide (COS) is an oligosaccharide formed by chitin/chitosan being hydrolyzed by acids, enzymes, or both with the N-deacetylated form of chitin and linear polysaccharides with a variable degree of N-acetylation. Chitosan derivative COS is composed of glucosamine bound to oligosaccharide chains via glucosamine-oligosaccharide links, and it has a degree of polymerization less than 20%. These units also contain reactive functional groups, including the primary and secondary hydroxyl groups at the C-3 and C-6 position (Li et al., 2015), to which the N-glucosamine units are linked by glycosidic bonds at β (1-4) link and other modifications (Aranaz et al., 2021). In comparison with Chitosan, COS's solubility and dispersibility in water is better in the hydrolysate as chitosan's molecular weight and chain are lower (Mao et al., 2021). Also, COS is absorbed by the intestines, quickly enters the bloodstream, and affects organisms systemically. This is the opposite of high molecular weight chitosan.

COS exhibits a wide range of biological activities and different reaction conditions can lead to different physicochemical properties in COS preparation. A number of the biological activities of COS are determined by its physicochemical properties, such as the degree of deacetylation (DD), charge distribution, and chemical modifications, such as antioxidant, anti-inflammatory (Lee et al., 2009), anti-obesity (Lee et al., 2021), anticancer (Termsarasab et al., 2013), and immunostimulant effects (Vanneman & Dranoff, 2012).

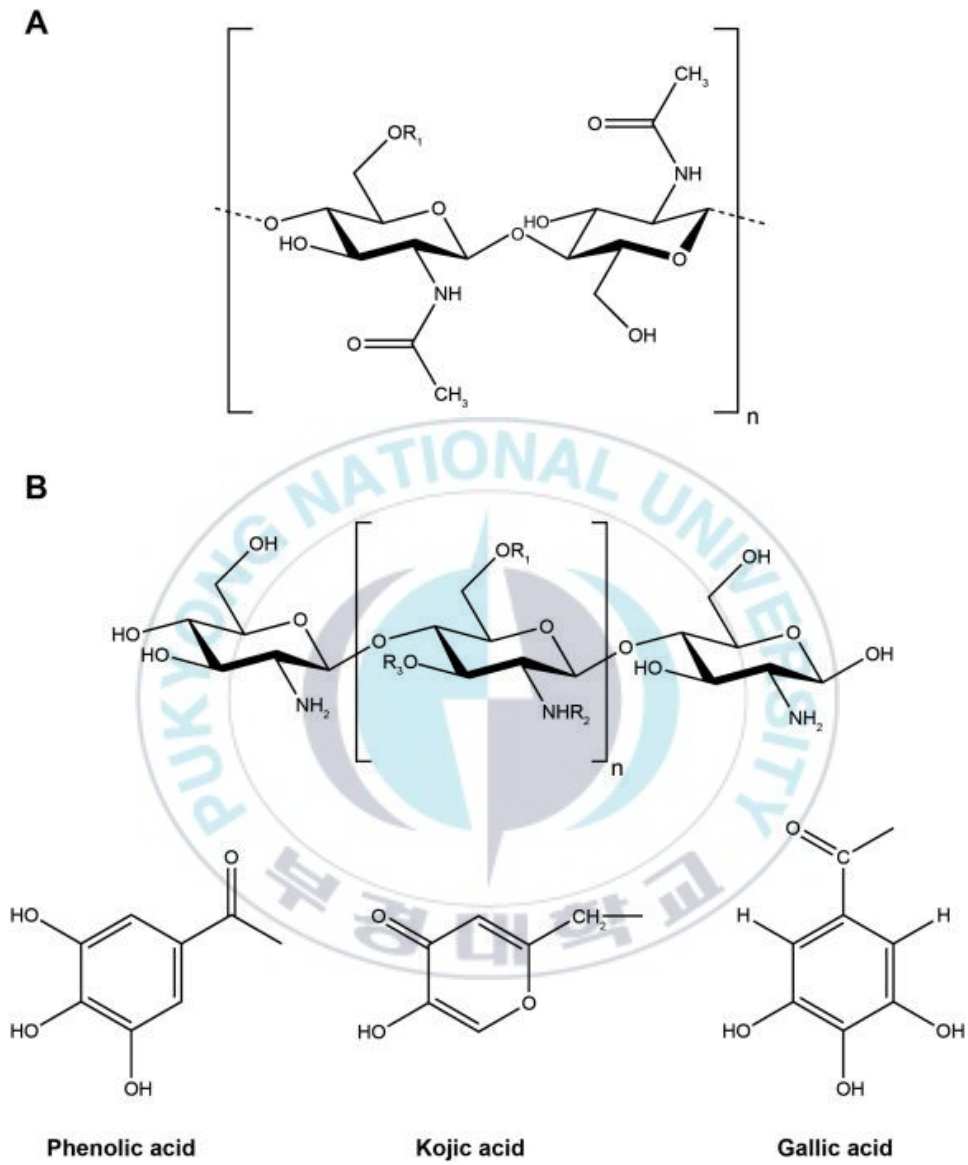

Figure 1. Chemical structures of chitin, chitosan, and chitosan oligosaccharide (COS)

Adapted from((Muanprasat & Chatsudthipong, 2017)

(A) Chemical structure of chitin and its derivatives.

(B) Chemical structures of chitosan, COS, and their derivatives

COS are positively charged because acetyl units have been removed from the d-glucosamine residues. Because COS molecules are positively charged, they can bind to microbial cell walls, thus inhibiting bacterial growth. COS's antimicrobial activity is favored by positively charged groups, so researchers expect chemical modifications of glucosamine's C-2 amino group will improve this activity (Jeon & Kim, 2001). COS has received a lot of attention because of its wide range of bioactivity and exhibits excellent antioxidant activity when it has been demonstrated to have significant anti-radical properties, albeit slightly less than ascorbic acid in some cases (Park et al., 2004). Besides that, many studies on the antimicrobial activities of chitosan (Jumaa et al., 2002). COS may alter permeability of the microbial cell membrane, further preventing the entry of materials or release of cell constituents, and finally lead to microbial death carried out a study confirmed this mechanism (Choi et al., 2001). In addition, the antimicrobial activity of COS depends on several factors, including the degree of deacetylation (JE et al., 2004). Additionally, COS binds to bacterial cell walls due to its positively charged nature. Polyelectrolyte compounds are formed when the positively charged amino group at the C-2 position of the glucosamine monomer interacts with the negatively charged carboxylic acid group of the macromolecules of the bacterial cell wall (Kim et al., 2003).

Furthermore, COS has been found to inhibit inflammation induced by LPS or other stimuli in a variety of cell types and animal models (Yoon et al., 2007). Injecting LPS into mice resulted in COS phosphorylation of the liver without phosphorylating JNK1/2 or p38. LPS-induced inflammation can be inhibited by

COS by inhibiting NF-κB and MAPK signaling pathways (Kim et al., 2014). This suppression of NF-κB translocation into the nucleus occurs through the inhibition of JNK 1/2 and IκB degradation by COS as a mechanism for suppressing LPS-induced inflammatory responses in macrophages. COS inhibits lymphocytes, basophils, and neutrophils as well. During the acute phase of inflammation, neutrophils and basophils are the primary granulocyte (Artis & Spits, 2015). Moreover, COS suppressed the NF-κB-induced activation of bovine melanin-associated antigens, TNF-α expression, and monocyte chemoattractant protein-1 expression in spleen-derived lymphocytes, suggesting that it inhibited lymphocyte activation as well (Kim et al., 2006).

Chitosan with different structures and physicochemical properties will produce different products, resulting in new bioactivities or a better understanding of known biocompatibility. As a consequence, COS holds a lot of promise in areas such as the environment, food, and pharmaceutical industries.

1.2 Foam cell formation

Atherosclerosis, a chronic disease characterized by excessive cholesterol deposition within the arterial intima, remains a leading cause of death worldwide. Developing severe atheromatous lesions is a sign of atherosclerosis, a chronic and progressive disease (Virmani et al., 2000). Atherosclerosis is most likely associated with a number of risk factors, including dyslipidemia, hypertension, diabetes, cigarette smoking, alcohol consumption, and lipoprotein modification (Rafieian-Kopaei et al., 2014). There is evidence that lipoprotein modification promotes atherosclerosis onset and progression (Mallat et al., 2010).

Among the various pathological factors, foam cell formation, which results from an imbalanced cholesterol influx and efflux, plays a critical role in the occurrence and progression of atherosclerosis. Excess cholesterol accumulates in peripheral cells, particularly macrophages and endothelial cells, under atherogenic conditions, as well as adhesion between macrophages disrupts cholesterol intrigants, esterification, and efflux, leading to foam cell formation (Mallat et al., 2010). The level of cellular cholesterol fluctuates because macrophages cannot regulate reverse cholesterol transport (Takeuchi & Akira, 2010). When atherosclerosis develops, cholesterol esterification and efflux become insufficient. Correspondingly, inhibiting cholesterol influx and promoting efflux both contribute significantly to the reduction of foam cell formation. In atherosclerosis, lipid uptake increases while cholesterol esterification decreases. The presence of foam cells is visible even in the early stages of atherosclerosis, and the accumulation of foam cells in advanced lesions is more common.

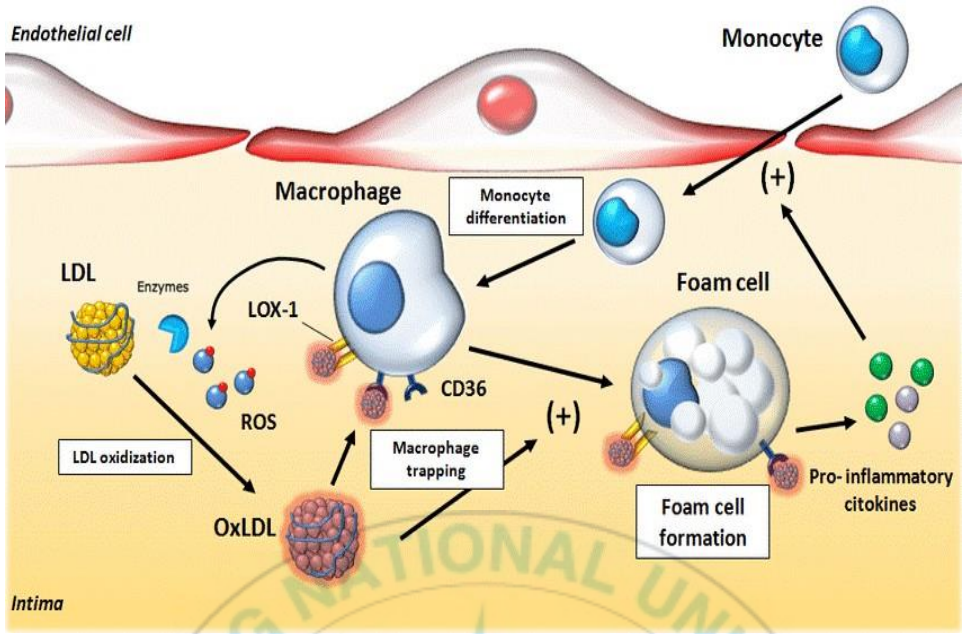

Figure 2. Foam cell formation mechanism by oxLDL (Adapted from: (Leiva et al., 2015)

Because macrophages are differentiated cells derived from monocytes, foam cells are expected to have a definite lifespan and could be replaced by other foam cells as atherosclerosis progresses. Although macrophages are changing into foam cells, they are poorly understood. Foam cells harbor lipid droplets that regress when apolipoprotein A-I or HDL is present in adequate amounts, indicating that lipid droplets are not for storing excessive amounts of lipid, but rather they are metabolically active (Maguire et al., 2019). Cholesterol efflux is controlled by over expression of several proteins, including ATP-binding cassette, sub-family A1 (ABCA1), and sub-family A1 (ABCA1) (Zhang et al., 2014). CD36, SR-A1, and SR-A2 are primary scavenger receptors that bind and uptake ox-LDL into macrophages (Yu et al., 2013). As a result, drugs that can regulate cholesterol flow and prevent cholesterol accumulation in macrophages may be useful in the treatment of atherogenesis. Specialized cells, such as macrophages, maintain low-density lipoprotein (LDL) and cholesterol homeostasis in the peripheral blood.

Low-density lipoprotein (LDL) and cholesterol homeostasis in the peripheral blood is maintained by specialized cells, such as macrophages. These include the scavenger receptor (SR)-A1, CD-36, and lectin-like oxLDL receptor-1 (LOX-1), which interact with lipoproteins. Several cholesterol transporters are present in these cells, including ABCA1, ABCG1, and SR-BI, which are involved in reverse cholesterol transport. Mechanisms of foam cell formation in atherosclerosis. In addition to transporting lipids and other metabolites across membranes, ABCA1 and ABCG1 rely on ATP as a source of energy. The human ATP-binding cassette (ABC) transporter superfamily. Furthermore, oxLDL-CD36 interactions

negatively impact macrophage migration by precluding cell polarization (Park et al., 2012). Therefore, oxLDL is implicated in not only macrophage activation and differentiation, but also macrophage retention.

Inflammation serves as the body's natural response to danger as a defense mechanism and one of the protective responses that lead to the activation of various enzymes and signaling proteins in stimulated cells such as macrophages, mononuclear phagocytes, and neutrophils (Ahn et al., 2016). The inflammatory response is triggered by harmful stimuli and conditions such as infection, toxins, and tissue injury. The chronic inflammation that occurs in the body plays a critical role in the development of a wide range of diseases, including atherosclerosis, obesity, asthma, arthritis, cancer, and autoimmune diseases (Medzhitov, 2008). Chronic inflammatory diseases are affecting people worldwide adversely and severely affecting their lifestyle, health, and well-being, according to the World Health Organization (WHO) (Pahwa et al., 2018) Activation of the immune system plays a crucial role in the development of inflammation.

A key regulator of inflammation and oxidative stress related molecules, such as ROS and SOD, is nuclear transcription factor-E2-related factor 2 (Nrf2) (Surh & Na, 2008). Heme oxygenase-1 (HO-1) is an enzyme that is regulated by Nrf2 and also regulates intracellular levels of ROS (Cheng et al., 2010). RAW264.7 macrophage cells and BV-2 cells were shown to have reduced apoptosis and inflammation by activating Nrf2 and HO-1 protein expression (Liu et al., 2002; Zhou et al., 2018).

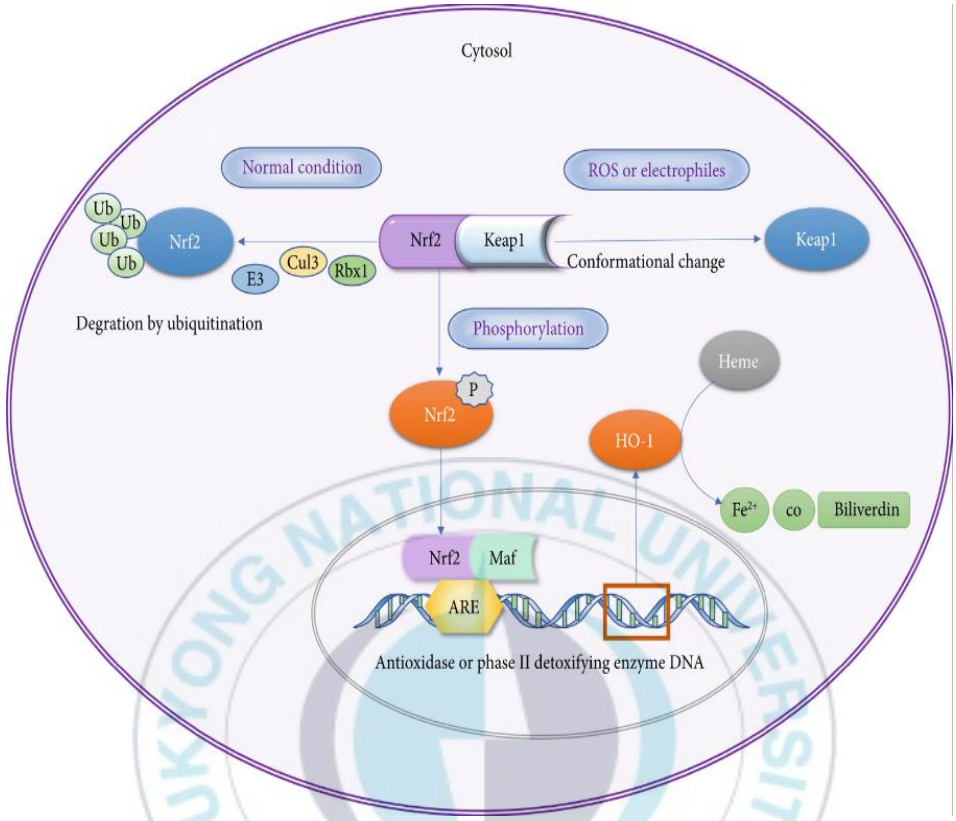

Figure 3. Nrf2/HO-1 pathway (Adapted from:(Zhang et al., 2019))

Thus, there is a possibility of preventing and treating a variety of oxidative stress-related and inflammatory diseases by activating the Nrf2/HO-1 signal pathway. ERK, JNK, and p38 MAPK comprise an important nuclear translocation of Nrf2 by mitogen-activated protein kinase (MAPKs). The activation of upstream signaling molecules MAPKs signaling pathways can modulate HO-1 and Nrf2 expression, according to recent studies (Choi et al., 2016).

Additionally, ERK1/2 and JNK1/2 function as downstream mediators of MAPK in addition to p38 MAPK. A mediator translocated activator protein 1 (AP-1) into the nucleus, which enhances the expression of proinflammatory genes. COS has been found to inhibit inflammation induced by LPS or other stimuli in a variety of cell types and animal models (Yoon et al., 2007). In in vitro and in vivo experiments on COS, it was demonstrated that a dose range of 20 μg/mL to 1 mg/mL caused anti-inflammatory effects, and 10 mg/kg/day to 100 mg/kg/day generated anti-inflammatory effects, respectively (Lee et al., 2009).

2. Materials and methods

2.1 Materials

COS (an average molecular weight of 3.5 kDa and 90% degree of deacetylation) were kindly donated by Kitto Life Co. (Seoul, Korea). A 2′,7′- Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Cell culture media Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, USA) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were analytical grade.

2.2 Cell culture

RAW264.7 macrophage (American Type Culture Collection USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 4 mM d-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were grown in humidified air at 37°C with 5% CO2.

2.3 Cytotoxicity assay

The cytotoxic effects of COS on RAW264.7 macrophages were studied with an MTT assay. Cells were seeded onto 96 well plates at a density of 3 x 10^5 cells/well and incubated for 24 h. Cells were treated with COS (1~5 mg/mL) in response to oxLDL (50 μg/mL). To each well, a solution of MTT (1 mg/mL) was added and

incubated for 4 hours. In viable cells, DMSO was used to dissolve formazan crystals and measure their absorbance at 540 nm.

2.4 Preparation of oxidized LDL and determination of thiobarbituric acid-reactive substances (TBARS)

For oxidation, LDL (1 mg/mL) was diluted in PBS and incubated at 37° C for 4 hours with 10 M $CuSO_4$ (final concentration). EDTA was added immediately to 30 µL of 1 mM to stop the process. The TBARS assay was used to determine the extent of LDL oxidation. In a ratio of 1:1, TCA (25% w/v) and TBA (1% w/v in 0.3% NaOH) were added to the reaction mixture, which was then boiled for 40 minutes at 92°C in the dark. Following completion of the reactions, an absorbance at 532 nm was determined using a microplate reader (MultiskanTM GO, Thermo Fisher Scientific, Rockford, IL, USA). A malondialdehyde (MDA) standard curve was used to determine the extent of LDL modification.

2.5 Oil red O staining assay

To determine lipid staining, oil red O staining was used. After COS (1~5 mg/mL) treatment followed by 1 hour of 50 µg/mL oxLDL. After 24 h cell was incubated with paraformaldehyde (4%) for 1 hour at room temperature and isopropanol (60%) for 5minute fixation. Working solution of Oil Red O was made from a stock solution (0.5 g Oil Red O in 100 mL isopropanol) by adding 3 mL of stock to 2 mL of distilled water. It was filtered through filter paper after it had been mixed well. After carefully removing the fixing buffer, cells were covered with fresh Oil Red O working solution for one hour at room temperature. In the next step, the rinsing of the cells was carefully rinsed with PBS (1 X) 2 times before air drying.

Image capture was performed using an inverted microscope (DMI6000, Leica, Wetzlar, Germany), and the absorbance was measured using a microplate reader at 510 nm (Multiskan™ GO, Thermo Scientific™, Waltham, MA, USA).

2.6 Determination of intracellular cholesterol content

Using a commercially available quantitation kit (Cholesterol/Cholesteryl Ester Quantitation Colorimetric Kit II; BioVision, Inc., Mountain View, CA, USA), the manufacturer's instructions were followed to determine the intracellular cholesterol and cholesteryl ester concentrations. Cells were treated with different concentrations of COS (1~5 mg/mL) for one hour, followed by oxLDL (50 μg/mL) for 24 hours. BCA was used to determine the protein content of each sample

2.7 Cellular cholesterol influx experiment.

The cellular cholesterol influx assay was performed using 25-NBD cholesterol. Cells were seeded in a 96-well clear-bottom black plate at 3 x 10^5 cells/well. Following by treated with COS (1~5 mg/mL) for 1 hour before being treated with oxLDL (50 μg/mL) for 24 hours. The media was removed, and the cells were labeled with 25-NBD 18 cholesterol (5 μg/mL) in serum-free DMEM for 6 hours. The amounts of cholesterol in the medium and cells were determined by using a microplate reader (GENios, TECAN, Männedorf, Switzerland) at 485 nm and 535 nm wavelengths.

2.8 Cellular cholesterol efflux experiment.

Following treatment as above, 25-NBD cholesterol-labeled, cells were washed with PBS and incubated in DMEM medium for another 6 hours. 25-NBD

cholesterol-labeled cells were washed with PBS and incubated in DMEM medium again for 6 h afterward. Fluorescence-labeled cholesterol was detected using a GENios microplate reader (GENios, TECAN, Männedorf, Switzerland) at excitation (485 nm) and emission (535 nm). As a percentage of the total fluorescence (the combined fluorescence of cells and the medium) cholesterol efflux was analyzed.

2.9 Determination of intracellular ROS formation using DCFH-DA labeling

The intracellular ROS formation was measured by using the oxidation sensitive dye DCFH-DA as a substrate (Engelmann et al., 2005) based on the report (Lee et al., 2013). RAW264.7 macrophages (3×10^5) were seeded in a black 96-well plate for 24 hours. Cells were treated for one hour with different concentrations of COS (1~5 mg/mL), followed by oxLDL (50 μg/mL) for 24 hours, and then for 20 minutes with 20 DCFH-DA in Hank's Balanced Salt solution (Thermo Fisher Scientific). The intensity of fluorescence was measured at excitation (485 nm) and emission (528 nm) wavelengths using a GENios microplate reader (GENios, TECAN, Männedorf, Switzerland) and an inverted fluorescence microscope (Leica, Wetzlar, Germany).

2.10 Determination of pro-inflammatory cytokines

The level of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) was quantified using BioTrak™ ELISA kits (GE Healthcare) according to the manufacturer's instructions after cells were treated with COS (1~5 mg/mL) for 1 hour followed by the addition of oxLDL (50 μg/mL) for 24 hours.

2.11 Western blot analysis

To prepare whole cell lysates, COS was applied to cells exposed or non-exposed to oxLDL. The RIPA buffer was supplemented with protease and phosphatase inhibitors. BCA assay kit (Thermo Scientific, Inc.) measured the protein concentration. Cell lysates (20 μg protein) were separated by 8 – 12% SDS-PAGE and transferred to PVDF membranes. PVDF membranes were blocked for 1 hour with 5% skim milk in TBS with 0.1% Tween 20 (TBS-T). Following that, the primary monoclonal antibodies (CD-36, SR-A, ABCA-1, ABCG-1, HO-1, iNOS, COX-2 and Nrf2) were mixed with 5 % skim milk in TBS-T and incubated at 4 °C overnight. The secondary antibody was used to detect the protein bindings in 2 hours. The ECL assay kit (Amersham Pharmacia Biosciences) was used for chemiluminescence detection, and the bands were imaged on a Davinch-Chemi imagerTM. By analyzing the level of actin protein, the basal levels of the proteins were normalized.

2.12 Statistical analysis

In this study, the data were analyzed using Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) and expressed as means + SD (n=3). Statistical significance was determined by P values < 0.05.

3. Results

3.1 Effect of COS on cytotoxicity in RAW264.7 macrophages

The MTT assay was used to check the cytotoxicity of the COS (1-5 mg/mL) concentrations did not have a cytotoxic effect on RAW264.7 macrophages. As a result of Figure 4, future analyses will be conducted using these concentrations.

3.2 Effect of COS on Oil red O staining assay

To determine the cellular lipid accumulation, an Oil red O assay was performed. As shown in Figure 5, the Oil red O staining area was significantly increased in RAW264.7 cells treated with COS followed by 1 hour of 50 μg/mL oxLDL, indicating that oxLDL effectively induces foam cell formation. By contrast, when COS (1 to 5 mg/mL) was added to oxLDL-induced foam cells, lipid accumulation was significantly reduced. The quantitative data showed that the treatment with oxLDL increased foam cell formation more than twofold, while the COS (5 mg/mL) treatment reduced it by 50%. As a result, COS demonstrated an inhibitory effect on foam cell formation.

3.3 Effect of COS on total cholesterol, free cholesterol and cholesterol ester content.

To assess the extent to which COS can inhibit foam cell formation, the amount of total cholesterol, free cholesterol, and cholesteryl ester content within the cell membranes was measured. As shown in Figure 6, COS treatment suppresses total cholesterol, free cholesterol, and cholesterol ester content within oxLDL stimulated RAW264.7 macrophages in dose-dependent manner.

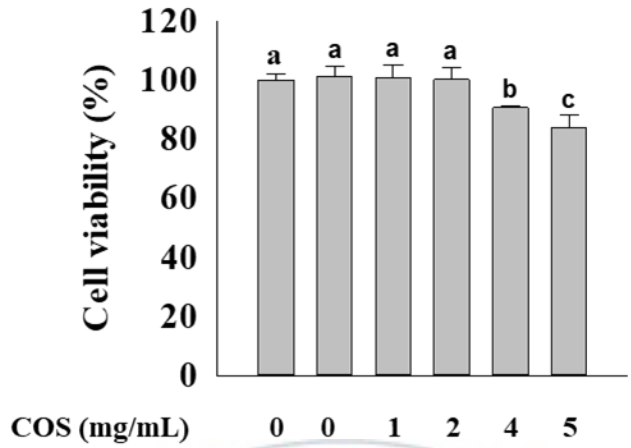

Figure 4. Cell viability in RAW264.7 macrophages. Cells were treated with COS for 24 h. Three independent experiments (n=3) showed a statistically significant difference, with error bars denoted by different letters ($P < 0.05$)

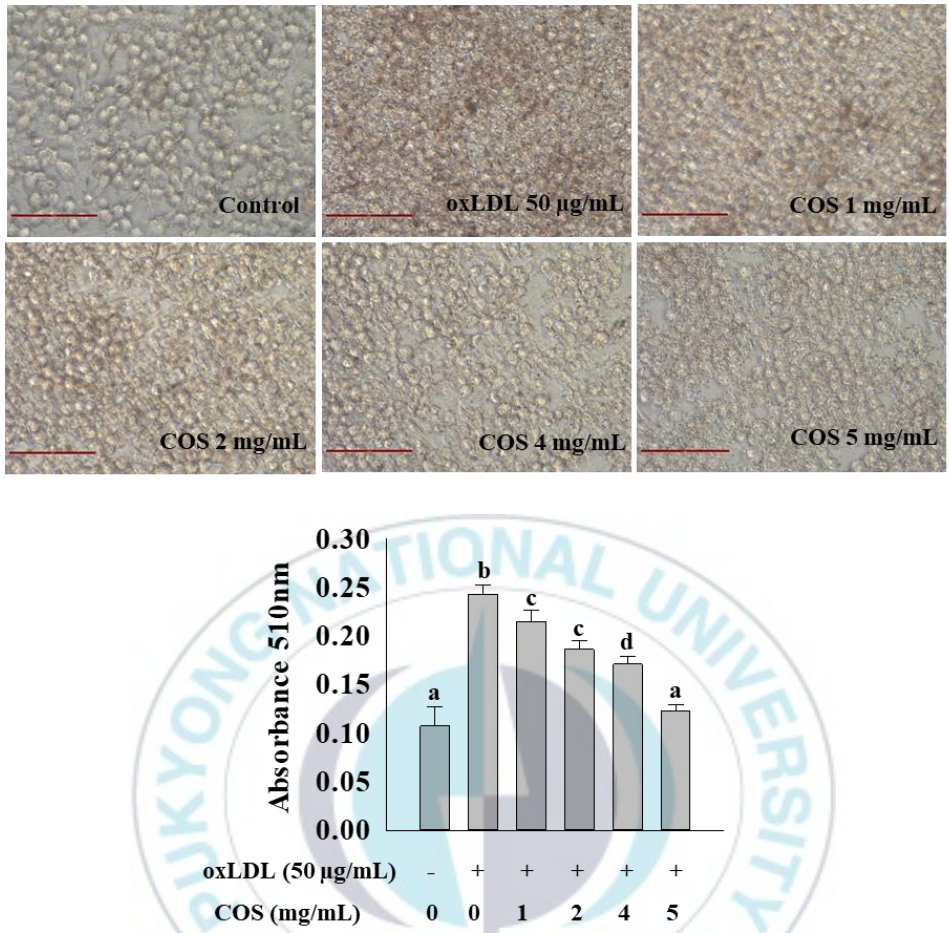

Figure 5. COS inhibits foam cell formation in RAW264.7 macrophages stimulated with oxLDL. Cells were first treated with COS for 1 hour, then with oxLDL for 24 hours. Three independent experiments (n=3) showed a statistically significant difference, with error bars denoted by different letters ($P < 0.05$).

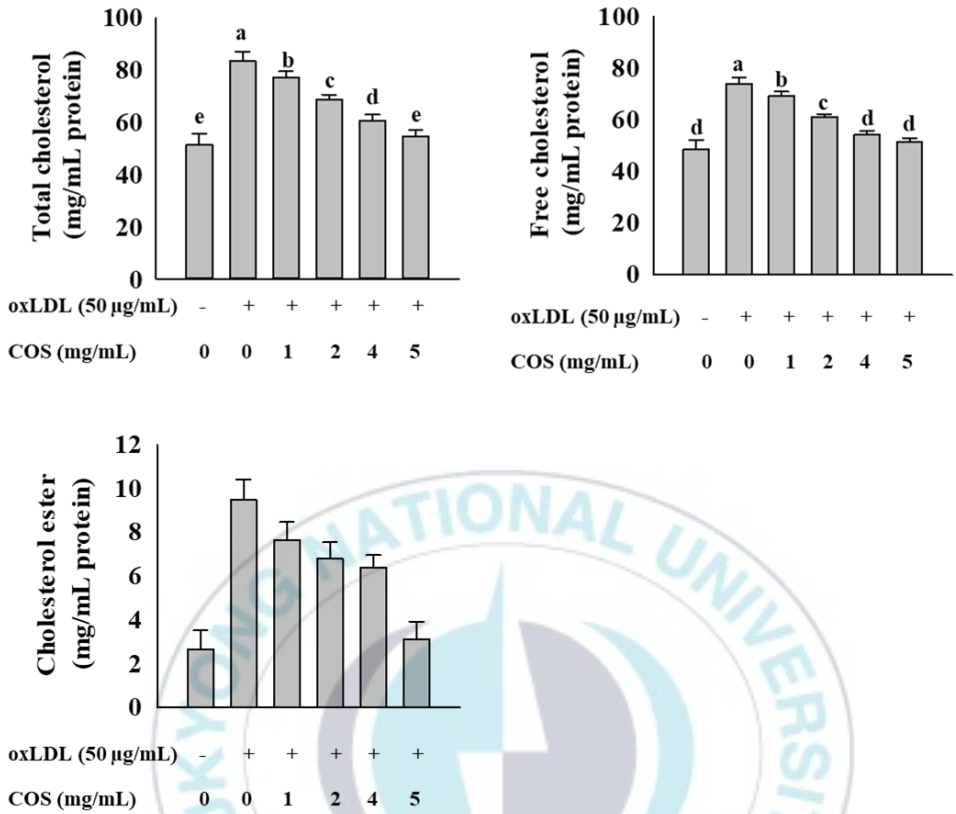

Figure 6. Effect of COS on total cholesterol (A), free cholesterol (B), and cholesteryl ester (C) content in oxLDL stimulated RAW264.7 macrophages. Cells were treated with COS for 1h followed by oxLDL treatment for 24 h. Three independent experiments (n=3) showed a statistically significant difference, with error bars denoted by different letters ($P < 0.05$)

In comparison to oxLDL treatment, the highest concentration of COS (5 mg/mL) resulted in a 34% reduction in total cholesterol, 30% reduction in free cholesterol, and 67% reduction in the content of cholesterol ester.

3.4 Effect of COS on cholesterol influx and efflux

Foam cells are formed as a result of disruptions in cholesterol influx, esterification, and efflux during the adhesion of macrophages. Since macrophages do not control the reverse cholesterol transporter, the cellular cholesterol level cannot be maintained at a constant level (Takeuchi & Akira, 2010). Therefore, cholesterol influx and cholesterol efflux were investigated in the presence of COS. The results shown in Figure 7 indicate that COS decreased cholesterol influx and increased cholesterol efflux in dose-dependent manner.

3.5 Effect of COS on cholesterol regulatory protein: ABCG-1, ABCA-1, SA-A1, and CD-36 expression.

The ability of oxLDL to cause cholesterol regulatory protein CD-36 to be expressed in macrophages was studied in order to investigate the mechanisms of COS. This was done by measuring the levels of SR-A, ABCA-1, and ABCG-1 expression in macrophages. By following Figures 8 and 9, it can be seen that the expression of CD-36 and SR-A1 were reduced by COS treatment in a concentration-dependent manner. Mean white, the expression of ABCA-1 and ABCG-1 genes increase after treatment with COS.

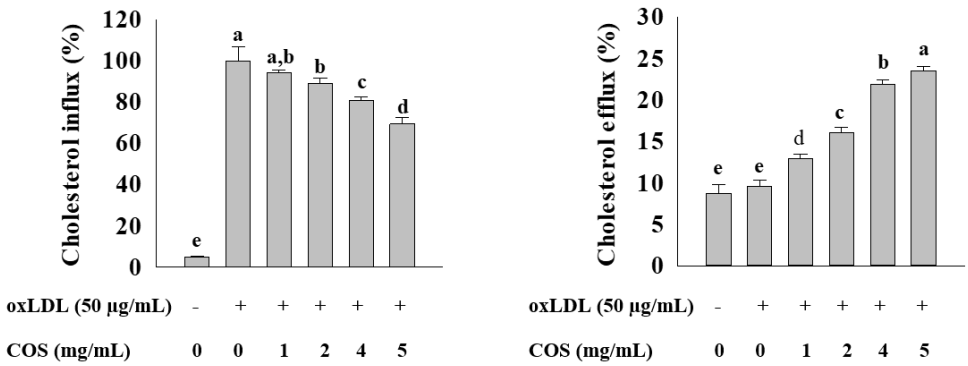

Figure 7. COS suppresses cholesterol influx (A) and efflux (B) in oxLDL-stimulated RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

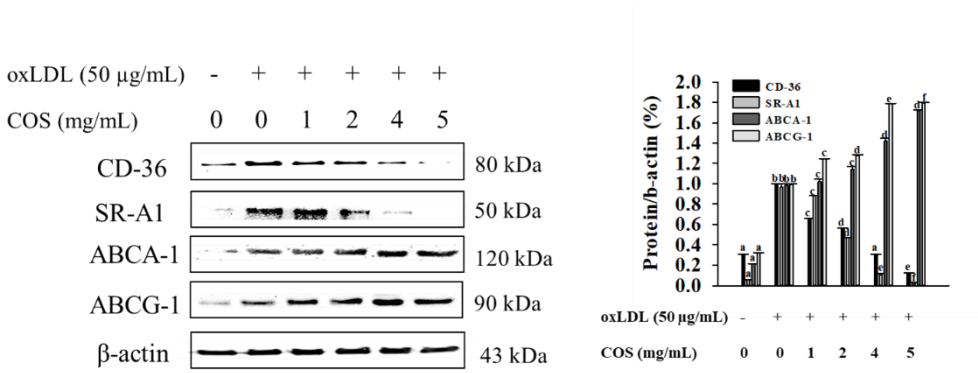

Figure 8. Effect of COS on CD-36. SR-A1, ABCA-1, and ABCG-1 protein expression in oxLDL stimulated by RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

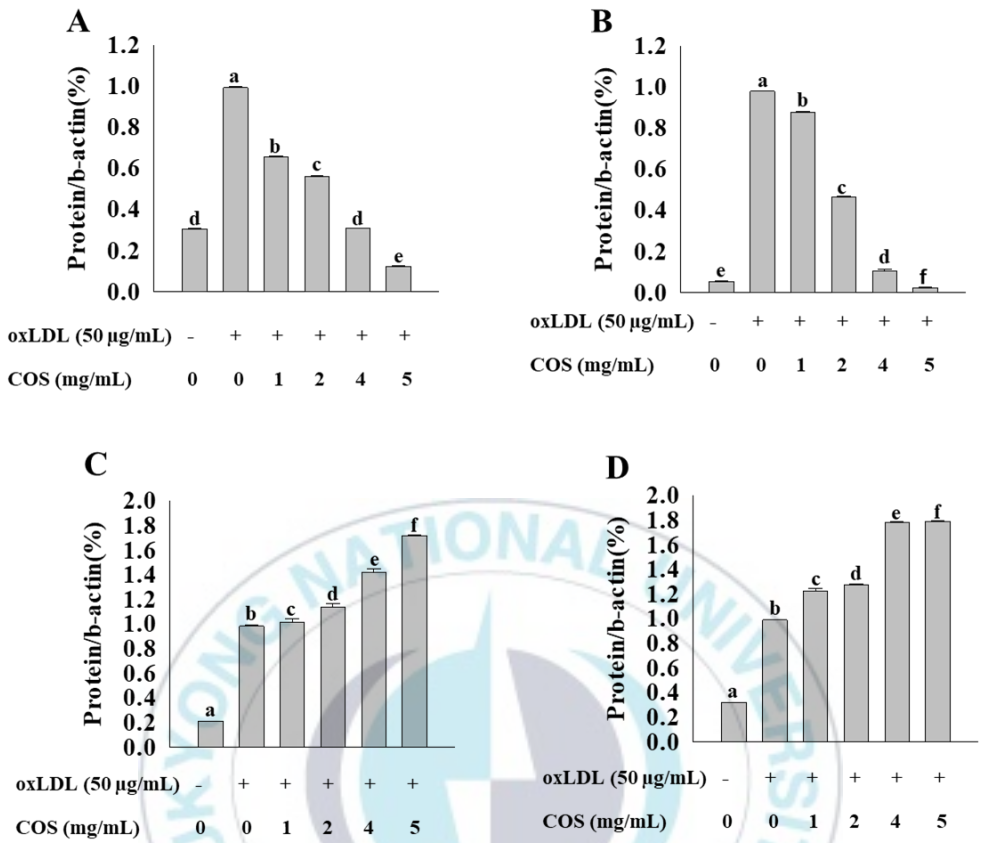

Figure 9 The percentage of protein expression of CD-36 (A). SR-A1 (B), ABCA-1 (C) and ABCG-1 (D) in oxLDL stimulated by RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

3.6 Effect of COS on pro-inflammatory cytokine production

The effect of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 on oxLDL-induced macrophages was studied by using an ELISA assay. COS treatment, as shown in Figure 10, reduced pro-inflammatory cytokine production in a concentration-dependent manner. However, when compared to the other two pro-inflammatory cytokines, COS treatment reduced TNF-α production the most. TNF-α, IL-1, and IL-6 levels in culture media were reduced by 71%, 59%, and 42%, respectively, after treatment with COS (5 mg/ml). These findings imply that COS treatment may regulate anti-inflammatory activities in RAW264.7 cells.

3.7 Effect of COS on ROS measurement

ROS production plays a key role in the progression of chronic inflammatory disorders due in part to chronic and prolonged intracellular ROS (Mittal et al., 2014). All along the labeling process, non-fluorescent DCFH-DA dye is hydrolyzed into DCFH, and DCFH is entrapped by intracellular esterase inside cells. ROS oxidized DCF to DCF during the subsequent oxidation of DCFH to DCF, which produces fluorescence. Figure 11 depicts intracellular ROS formation in the presence of COS. DCF emitted fluorescence after ROS-mediated oxidation of DCFH under different concentration measurements. As compared to macrophages not treated with oxLDL, those treated with oxLDL showed a significant decrease in fluorescence intensity. The concentration-dependent decrease in fluorescence intensity slowly decreased in cells treated with COS, with 5 mg/ml COS showing a 66% reduction in fluorescence intensity versus cells treated with oxLDL.

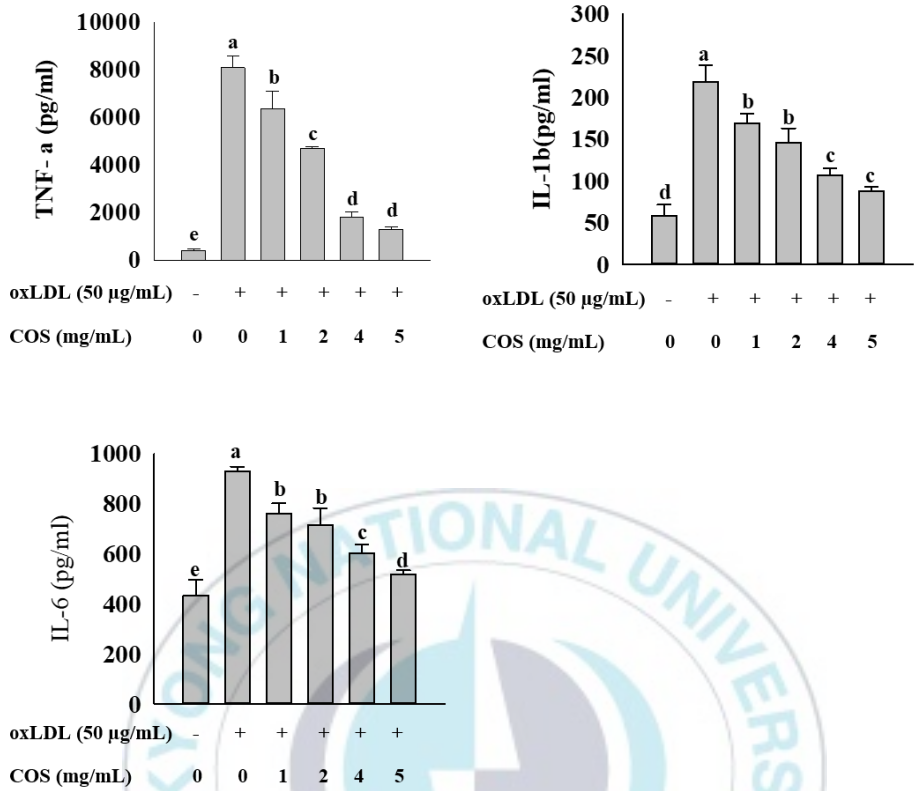

Figure 10. The effect of pro-inflammatory cytokines: TNF-α, IL-1β, and IL-6 in oxLDL stimulated by RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

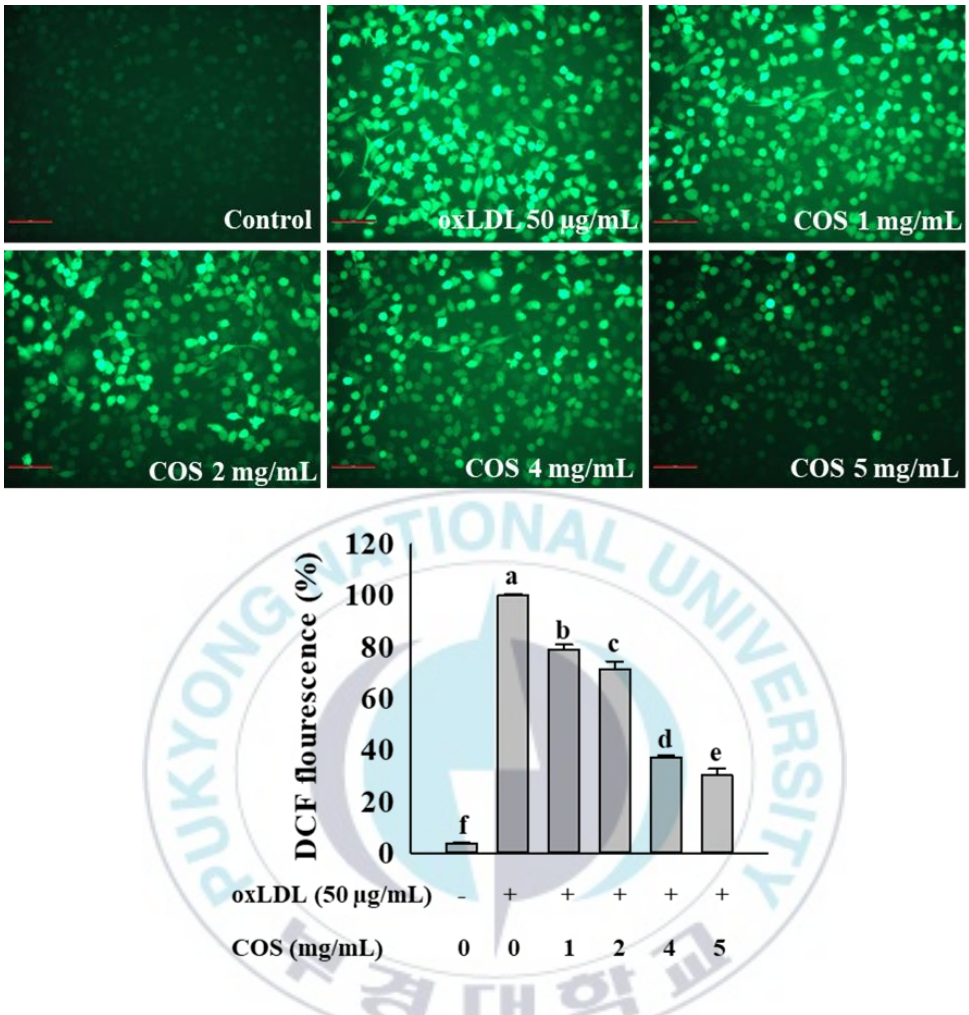

Figure 11. COS inhibits the intracellular generation of ROS in RAW264.7 macrophages. The oxidation-sensitive dye DCFH-DA was used to measure intracellular ROS formation. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

3.8 Effect of COS on HO-1, COX-2, and iNOS expression in oxLDL-stimulated RAW264.7 macrophages.

COS inhibited the production of NO and PGE2 in macrophages stimulated with oxLDL by inhibiting the expression of corresponding genes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The effects of COS on macrophage protein expression were examined by Western blot analysis. According to the Figures 12 and 13, the expression of iNOS and COX-2 significant decrease with COS treatment in dose-dependent manner, it showed appreciate 1.5-time and 5.4-time reduction at the highest concentration of protein expression level of iNOS and COX-2 respectively. On the other hand, in the 5 mg/mL COS treatment followed by the oxLDL stimulation, more than 2.5 times the level of inhibition was seen, it dramatically increases HO-1 expression in a dose-dependent manner compares to the ox-LDL only treatment.

3.9 Effect of COS on Nrf2 nuclear translocation

The translocation of Nrf2 to the nucleus is the primary signaling pathway that leads to the induction of HO-1. Hence, Nrf2-mediated HO-1 expression by COS was examined in murine macrophages to better understand how it functions. As shown in Figure 14, HO-1 induction was mediated by increased nuclear Nrf2 translocation by COS treatment in contrast to non-treatment, indicating that Nrf2 translocation by COS led to HO-1 induction.

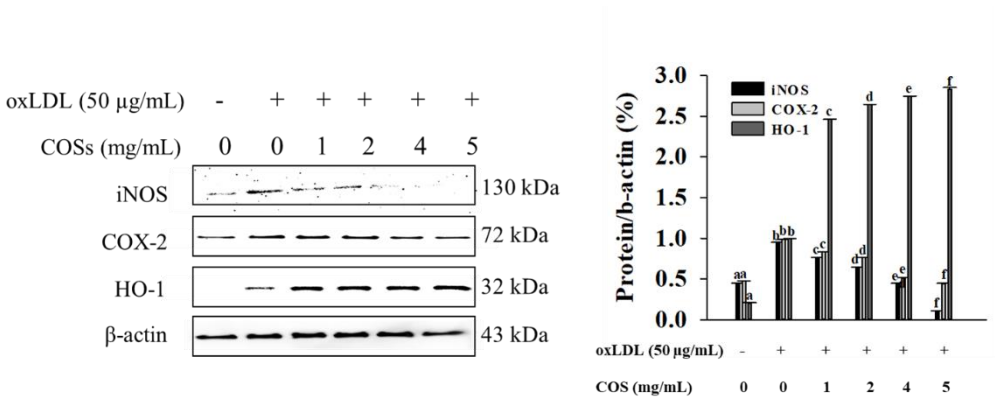

Figure 12. Effect of COS on HO-1, COX-2, and iNOS protein expression in oxLDL stimulated RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

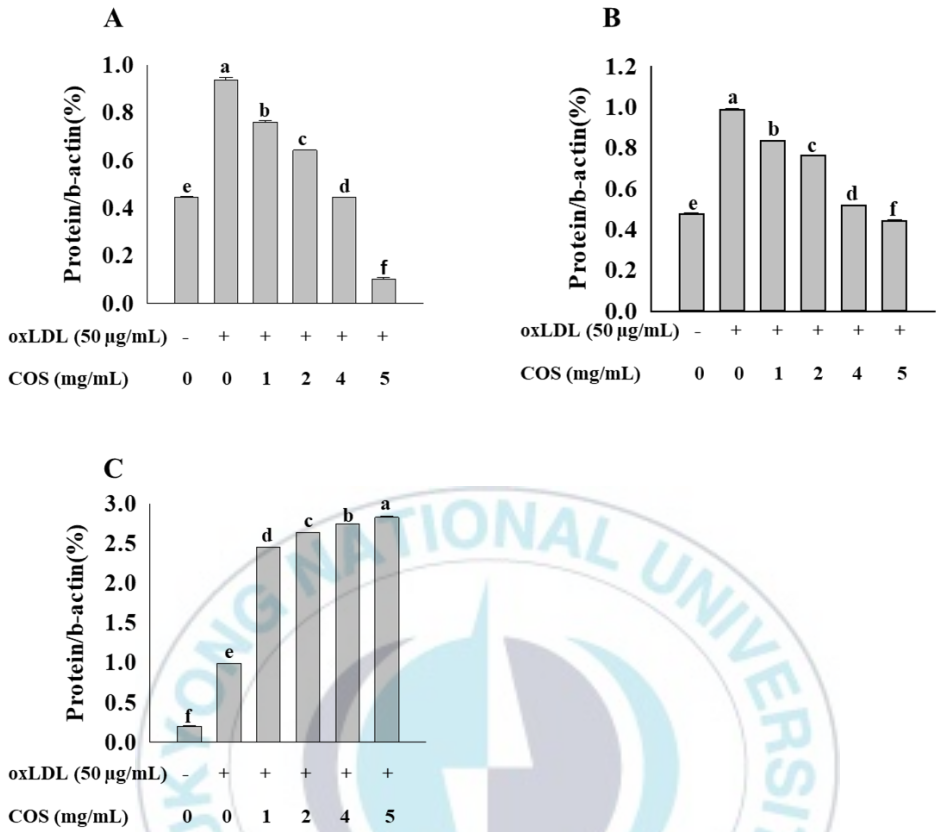

Figure 13. The percentage of protein expression of iNOS (A), COX-2 (B), and HO-1 (C) in oxLDL stimulated RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

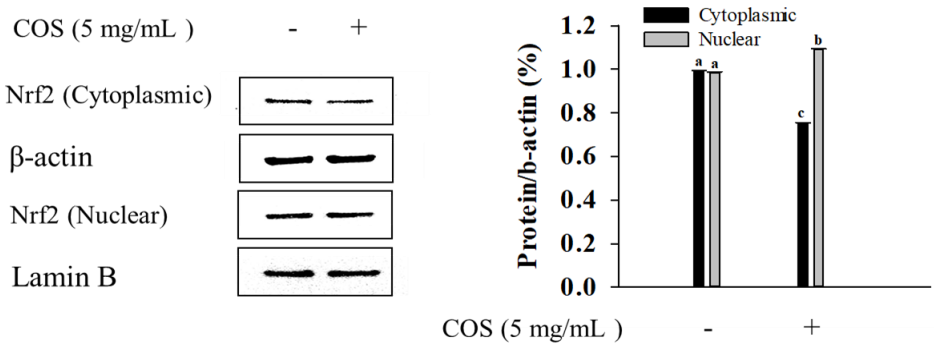

Figure 14. Effect of COS on Nrf2 in oxLDL - stimulated RAW264.7 macrophages. Cells were treated with different concentrations of COS for 1h followed by treatment with oxLDL for 24 hours. A statistically significant difference was found between three independent experiments (n=3) with error bars denoted by different letters being significantly different ($P < 0.05$).

4 Discussion

The degree of COS biological activity is given as the acetylation level with the acetamide group at position C-2 or as the degree of chitosan hydrolysis with the amino group. Several of the COS derivatives that are being used for medicinal purposes contain reactive amino and hydroxyl groups (Kim et al., 2005). It is common for COS to have a Mw of < 10,000 Da and a DP between 50 and 55 (Vishu Kumar et al., 2007). Despite this, there are no restrictions on their research or application. A systematic analysis of COS with varying MW and DD (80 & 90) was conducted along with its antibacterial activity (Laokuldilok et al., 2017) at the lowest effective concentration, COS with a MW of 5.1 kDa had the greatest effects against DPPH radicals.

Due to their relatively easy processing, several COS derivatives can be produced under these conditions, and there is a positive correlation between COS's deacetylation level, molecular weight, and anti-inflammatory properties in vitro. The in vivo absorption of COS by the intestinal tract was also decreased by increased MW when COS with MW 3.8 kDa was eaten orally at a concentration of 20 mg/kg (Chae et al., 2005). The charge distribution of COS makes it easier to absorb via intestinal epithelium due to its favorable MW. Additionally, this dietary supplement is biocompatible (Liu et al., 2013) non-allergenic (Azuma et al., 2015), mucoadhesive (Lee et al., 2017), and easily absorbed throughout the digestive tract (Liu et al., 2007). In order to exploit COS in its oligosaccharide form, numerous studies have been conducted on it.

According to previous studies, COS exerted anti-inflammatory effects on LPS-stimulated murine macrophages by up-regulating HO-1. By phosphorylating MAPKs, Nrf2 is transported to the nucleus and the expression of HO-1 is up-regulated (Hyung et al., 2016). Several studies have been conducted to study chitosan's anti-inflammatory properties, including its ability to modulate inflammatory proteins and pro-inflammatory cytokines. In spite of a lack of information, the anti-inflammatory properties of COS given to oxidized low-density lipoprotein-stimulated murine macrophages are unknown. Thus, we determined oxidized low-density lipoprotein in RAW264.7 cells at different concentrations to examine the inhibitory effect on foam cell formation and the anti-inflammatory activities through the HO-1/Nrf2 pathway.

In the body, foam cells are a type of macrophage that can cause atherosclerosis, heart attacks, and strokes because they accumulate in the bloodstream. The modified lipoproteins are internalized by the macrophages in the subendothelial space after they have differentiated into monocytes (Yuan et al., 2012). Atherosclerosis begins with the formation of foam cells caused by the oxidative modification of LDL. Instead, oxLDL promotes atherosclerosis by causing lipid dysregulation and foam cell formation, instead of promoting inflammation and immunity. The formation of foam cells is accompanied by an inflammatory response. Inflammation, oxidative stress, and inflammation associated with oxidative stress are some of the key components of chronic inflammation. oxLDL receptor one (LOX-1), the receptor that mediates oxLDL uptake, plays a central role in these processes (Pothineni et al., 2017). In macrophages, reactive oxygen

species (ROS) cause LDL to become oxidize LDL, and the oxLDL is scavenged and internalized by the SR-A1 and CD36 scavenging receptors (Kishimoto et al., 2010). Also, ROS and other reactive species can lead to altered redox systems, including thioredoxin reductase, glutathione peroxidase, and pyridine nucleotide biosynthesis, and affect normal cell signaling, including apoptosis (Zhang et al., 2013). As part of its pathogenic role, CD36 is also found to inhibit macrophage migration, the spreading of cells, and the activation of focal adhesion kinase through Src-kinase signaling and oxidative stress, as well as the internalization of oxLDL (Park et al., 2009). In addition, oxLDL-CD36 interaction is associated with macrophage polarization loss, which is an essential step in how cells migrate (Park et al., 2012).

Reverse cholesterol transport (RCT) is thought to be a very significant mechanism in macrophage survival that provides cholesterol efflux from foam cells. In macrophages, several cholesterol transport mechanisms play key roles in circulating cholesterol, for example, SR-B1, ATP binding cassette transporter A1 (ABCA-1), and ATP binding cassette transporter G1 (ABCG1) (Park et al., 2012). The component of apoprotein A1 (apoA1) is formed when a component of ABCA-1 is responsible for the release of cholesterol from the cells (Gelissen et al., 2006). It has been shown that ABAC-1 is an important factor in reverse cholesterol transport.

Accordingly, we measured cellular lipid uptakes by Oil Red O staining, total cholesterol, free cholesterol, and cholesterol ester content and cholesterol influx assays. This was done in order to determine whether or not the COS treatment can

effectively inhibit foam cell formation. Based on our results, COS treatment inhibits the formation of foam cells through reduction of the lipid droplets, reduction of total cholesterol, free cholesterol, and cholesterol ester content, and decrease of cholesterol influx by a dose-dependent mechanism. However, COS effect on cellular lipid metabolism was investigated by cholesterol efflux assay, and results showed increased cholesterol efflux dose-dependently.

In addition, we looked into the effects of SR-A1 and CD36 receptors on lipid accumulation in cells, as well as ABCA-1 and ABCG-1 on lipid outflow, by analyzing western blots. COS treatment inhibits the expression of SR-A1, CD36 as well as ABCA-1, while providing a significant increase in ABCA-1 and ABCG-1 are enhanced.

The purpose of this study was to investigate whether COS treatment can effectively be used to inhibit the formation of foam cells. Our results show that COS treatment decreased the effect of lipid droplets in Oil Red O staining as well as inhibiting the expression of SR proteins, including SR-A1 and CD36 and enhancing the expression of ABC transporters, including ABCA-1 and ABCG-1 as well as decreasing levels of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β in cell media. Thus, COS can be potentially preventing foam cells from forming as it inhibits the formation of foam cells.

OxLDL may cause the foam cells to apoptosis and/or necrotize, which may result in a buildup of debris in the core of the atherosclerotic plaque, which contributes to inflammation.

Macrophages are mainly responsible for the host's defense against cancer and infection. Conversely, chronic inflammation caused by endotoxins or inflammatory mediators may lead to inflammatory diseases and cancer because of excessive amounts of pro-inflammatory mediators and cytokines (Abarikwu, 2014). The development of anti-inflammatory agents can therefore be achieved by preventing macrophages from responding excessively to inflammation-related signals (Kim et al., 2016). Proinflammatory cytokines like TNF-α, IL-1β and IL-6 secreted by macrophages trigger the production of NO and the inflammatory, cytotoxic, and fever responses triggered by oxLDL stimulation. COS has been shown to be a potential increase in LDL-induced NO production, in addition to cytokines that aid in inflammation, such as TNF-α, IL-6, and IL-1β (Kim et al., 1999; Salvemini et al., 1993). By converting L-arginine to NO, iNOS converts L-arginine in the body into cytotoxicity and tissue damage as a result of inflammation. COX-2 produces arachidonic acid into PGE2 by converting it to acetate. Furthermore, PGE2 is also involved in metastasis and invasion of cancer cells, in addition to its function in inflammation (Lai et al., 2011). There is a significant amount of NO produced by iNOS within the inflammation process, which in turn is a consequence of its activation of secondary messenger molecules. By regulating PGE2 production, COX-2 also regulates cell proliferation, apoptosis, and immune response in the body. The presence of these two enzymes plays a direct role in inflammatory diseases, as they promote the production of cytokines. According to the results of the western blot analysis, COX-2 and iNOS were present in the samples. In the present study, COS was demonstrated to inhibit the

decrease in protein expression of COX-2 and iNOS in response to the oxidation of LDL-C by dose-dependent mechanisms.

There is no doubt that ROS play a crucial role in inflammatory diseases through modulating the inflammation response during the process of initiation, progression, and resolution. Furthermore, increased ROS production can cause endothelial dysfunction (Chelombitko, 2018). Also, it can also be caused by imbalances between production and elimination of ROS at the cellular level, which can also lead to oxidative stress (Hussain et al., 2016). As a result of cytokines and chemokines produced by inflamed cells, an accumulation of ROS is also caused by inflammation responses.

Consequently, COS was investigated by attenuating inflammation by inhibiting the production of pro-inflammatory cytokines and ROS as measures of its anti-inflammation effects. The anti-inflammatory effects of COS were demonstrated in reducing cytokines such as TNF-α, IL-1β, and IL-6, and ROS production during oxLDL-induced inflammation. Based on the results of this research, COS reduces several things, including iNOS, COX-2 expression, and the production of ROS-proinflammatory cytokines. This suggests that COS may have potential anti-inflammatory properties.

Transcriptional factor Nrf2 modulates HO-1 expression upstream and is a master regulator for the expression of HO-1. In a recent study, it was found that HO-1 upregulation is associated with a reduction in inflammation (Shie et al., 2016; Song et al., 2016). HO-1 activity increases in response to LPS as LPS inhibits the production of NO, iNOS, COX-2, and pro-inflammatory cytokines, as well as HO-

1 activity increases in response to LPS. The cytoplasmic Nrf2/Keap1 complex is released from the nucleus of the cell after the sequestered Nrf2 is activated. In addition to its function as a regulator, the liberated Nrf2 material also causes the expression of a number of target genes, including HO-1 (Kansanen et al., 2013). The Keap1 protein undergoes a conformational change under oxidative stress due to a modification of its cysteine residue and the release of Nrf2. Once it reaches the nucleus, it activates the antioxidant response element (ARE) resulting in the production of antioxidant gene expression as HO-1 (Loboda et al., 2016). A result of this study has shown that COS induces Nrf2 translocation into the nucleus through HO-1, and that this translocation is facilitated by HO-1. During foam cell formation, it is occurred inflammatory response too. COS suppress foam cell formation because COS inhibit foam cell formation, it can also be expected reduced inflammatory response.

5. Conclusion

Recently, studies have gained much attention to the properties of COS as naturally occurring biopolymer exhibits unique biological properties. The present study examined the effect of COS on foam cell formation inhibition and anti-inflammatory properties of RAW264.7 macrophages. It suppressed lipid accumulation and enhanced lipid outflow in foam cells by inhibiting foam cell formation. Likewise, COS treatment reduced the expression of SR-A-1 and CD-36 proteins while increasing ABCA-1 and ABCG-1 protein expression. Furthermore, COS inhibited inflammation by reducing NO production, ROS production, and pro-inflammatory cytokine production while increasing HO-1 expression and Nrf2 nuclear translocation. As far as we know, COS or chitosan cannot induce HO-1 in murine macrophages following the stimulation of oxLDL. Furthermore, this study provides a premise for determining whether HO-1 induction by COS contributes to anti-inflammatory activity as well as identifying the signaling pathways that facilitate this action.

In conclusion, these results suggest that COS may have the potential foam cell formation inhibition by regulating inflammatory response.

6. Abstract (Korean)

RAW264.7 대식세포에서 키토산올리고당의 oxLDL 의 의한 거품세포 형성 및 염증 반응 억제 효능

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요 약

키토산 올리고당(COS)은 키토산 또는 키틴에서 파생되며 항염, 항산화, 항암 등의 특성을 나타내는 것으로 설명되었습니다. 본 연구의 현재, COS 는 산화된 저밀도 지단백질(LDL)로 자극된 RAW264.7 세포에서 거품 세포 형성 억제 및 항염증 효과를 조사하는 데 사용되었습니다. COS 는 거품 세포 형성에 억제 효과가 있는 것으로 밝혀졌습니다. RAW264.7 대식세포의 ox-LDL 자극 동안, COS 는 콜레스테롤 유입과 SR-A1 및 CD-36 단백질 발현 수준을 감소시키면서 콜레스테롤 유출과 ABCA-1 및 ABCG-1 단백질 발현 수준을 증가시켰습니다. 그 외에도 COS 처리는 용량 의존적 방식으로 oxLDL 유도 RAW264.7 대식세포에서 핵인자-카파 B(NF-kB) 핵 전위를 억제합니다. 이러한 발견은 COS 가 거품 세포 형성에 억제 효과를 가질 수 있음을 의미합니다. 활성산소종(ROS)의 생성을 줄임으로써 COS 는 종양 괴사 인자 알파(TNF-α), 인터루킨-1(IL-1β), 인터루킨-6(IL6). 또한, oxLDL 로

자극된 RAW264.7 세포의 COS 는 헴-옥시게나제-1(HO-1) 발현을 증가시키는 반면 iNOS 및 COX-2 발현을 억제합니다. 또한, COS 는 핵 인자 적혈구계 2 관련 인자 2(Nrf2)의 핵 전위를 상당히 향상시킵니다. 이러한 발견에 따르면 COS 는 Nrf2/HO-1 경로를 활성화하여 보호 효과를 발휘할 수 있습니다.

핵심어: 키토산올리고당, 거품세포형성, 항염, oxLDL, RAW264.7 대식세포

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