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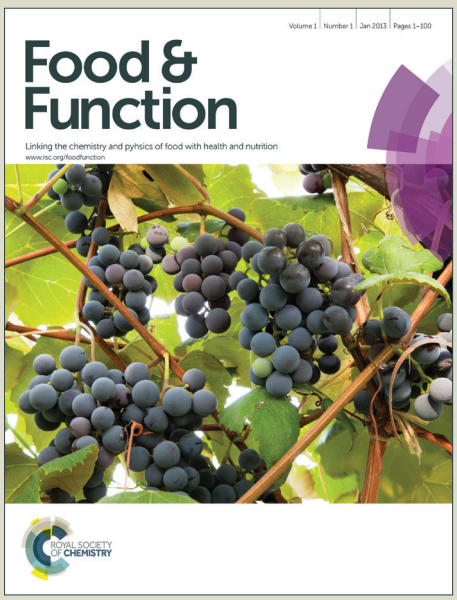

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Consumption of high-dose vitamin C (1,250 mg/day) enhances functional and structural properties of serum lipoprotein to improve anti-oxidant, anti-atherosclerotic, and anti-aging effects via regulation of anti-inflammatory microRNA

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Running head: vitamin C, lipoproteins, miRNA

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Abbreviations : apo, apolipoprotein; HDL, high-density lipoproteins; LDL, low-density lipoproteins; miR, microRNA; TG, triacylglycerol;

ABSTRACT

Background: Although the healthy effects of vitamin C are well known, its physiological effect on serum lipoproteins and microRNA are still remained to be investigated, especially daily consumption of high dosage.

Objectives: To investigate the physiological effect of vitamin C on serum lipoprotein metabolism in terms of its anti-oxidant, anti-glycation activities, and gene expression via microRNA regulation.

Methods: we analyzed blood parameters and lipoprotein parameters in young subjects (n=46, 22±2 years old) including smokers who consumed a high dose of vitamin C (1,250 mg) daily for 8 weeks.

Results: Antioxidant activity of serum was enhanced with elevation of vit C content in plasma during the 8 weeks consumption. In LDL fraction, apo-B_{48} band disappeared at 8 weeks post-consumption in all subjects. In HDL fraction, apoA-I expression was enhanced by 20% at 8 weeks, especially in male smokers. In the lipoprotein fraction, all subjects showed significantly reduced contents of advanced glycated end products and reactive oxidized species (ROS). Triglyceride (TG) contents in each LDL and HDL fraction were significantly reduced in all groups following the Vit C consumption, suggesting that the lipoprotein became to have more anti-inflammatory and atherogenic properties. Phagocytosis of LDL, which was purified from each individual, into macrophages was significantly reduced at 8-weeks post-consumption of vitamin C. Anti-inflammatory and anti-senescence effect of HDL from all subjects was enhanced after the 8-weeks consumption. Expression level of microRNA 155 in HDL_3 was reduced by 49% and 75% in non-smokers and smokers, respectively.

Conclusion: The daily consumption of a high dose of vitamin C for 8 weeks resulted in enhanced anti-senescence and anti-atherosclerotic effects via improvement of lipoprotein parameters and microRNA expression through anti-oxidation and anti-glycation especially in smokers.

Keywords: Vitamin C, lipoprotein, oxidation, glycation, anti-aging, microRNA

INTRODUCTION

Vitamin C (Vit C) is a strong antioxidant that exerts many beneficial functions for the prevention of diseases such as cancer (1), atherosclerosis (2), and aging-related diseases (3). Vit C is considered as an anti-aging molecule (4). Aging process has negatively effects on the human body, such as increased oxidative stress and chronic inflammatory response (5). Symptoms of aging are related to incidence of metabolic syndrome, including hypertension, hyperlipidemia, and diabetes (6). Many aging-related diseases are closely associated with serum lipid, cholesterol and triacylglycerol levels as well as lipoprotein metabolism. In blood, HDL-cholesterol (HDL-C) is inversely associated with incidence of aging-related diseases (7).

Incidence of cardiovascular disease (CVD) and diabetes is closely related with lipid metabolism and aging stress (8, 9). HDL-C is inversely correlated with incidence of CVD and has potent anti-oxidant and anti-inflammatory activities (10). However, HDL can be transformed into dysfunctional HDL, which is more atherogenic, via induction of aging stress such as oxidation and glycation (11, 12).

Smoking is a major stress source of HDL oxidation and glycation, resulting in loss of the beneficial functions of HDL. Recently, our research group reported that HDL from young smokers (22 ± 2 years old) contains a high amount of dysfunctional HDL (13), with higher proportions of glycated and oxidized species. Further, apoA-I from smoker HDL shows severe truncation and multimerization with smaller HDL particles. These changes are very similar to the functional and structural properties of HDL from the elderly, as we reported previously (14, 15).

However, the negative effects of serum HDL during disease can be reversed upon recovery to a healthy state. As we reported before (16), impairment of HDL structure and function in

oliguric phase can be reversed during recovery. Furthermore, athletes engaging in aerobic exercise (runners and wrestlers) exhibit enhanced antioxidant activities of HDL, increased apoA-I levels, and larger HDL particle size (17). Incorporation of oleic acid and w-3 fatty acid into reconstituted HDL has been shown to enhance anti-oxidant and anti-atherosclerotic functions of HDL (18). These results suggest that HDL quality can be enhanced by healthy activities such as exercise and intake of healthy foods.

As Vit C is a well known anti-aging ingredient, it may be possible that Vit C supplementation can enhance the beneficial functions of HDL in terms of anti-oxidant, anti-glycation, and anti-inflammatory activities. Furthermore, a high dose of Vit C (up to 20 μM) has been shown to mediate anti-cancer activity with pro-oxidant and cytotoxic effects in a tumor xenograft mice model (19).

Although many reports have determined the health beneficial effects of a low or normal dose of Vit C, high-dose supplementations (1,250 mg/day) of Vit C in young subjects and smokers have not been investigated. In the current study, to investigate physiological changes as well as functional enhancement of HDL upon Vit C supplementation, we analyzed serum lipoproteins from smokers at 8 weeks post-consumption of Vit C (daily 1,250 mg in tablet).

As a new function of lipoprotein, it was recently reported that microRNA (miRNA) is transported by lipoprotein carriers in the blood (20). The miRNA is a key post-transcriptional biological regulator of gene expression in cells. Circulating extracellular miRNAs have been discovered (21) and many studies have reported circulating miRNAs as stable blood-based markers for many diseases (22). Plasma miRNAs circulate and are transported to target cells via binding to lipoproteins such as LDL and HDL. MiR155, which is carried mainly by HDL,

is well characterized as an inflammatory regulator miRNA. The level of miR155 is induced by the macrophage inflammatory response, such as LPS and oxidative stress (23). In this study, the level of miR155 was compared among HDL isolated from smokers and non-smokers after consumption of the Vit C.

SUBJECTS and METHODS

Subjects and vitamin C supplementation

We recruited non-smoker female (NS-F, n=14, 20.3±1.0 years old), non-smoker male (NS-M, n=20, 21.3±1.5 years old), and smoker male (S-M, n=12, 24.0±1.7 years old) subjects of similar age living in Daegu city (South Korea). During 8 weeks, all participants consumed daily one tablet of Vitamin C (1,250 mg of vitamin C, Kwangdong pharmaceutical Co., LTD., Seoul, Korea). Informed consent was obtained from all participants prior to enrollment in the study, and the Institutional Review Board at the Yeungnam University (Gyeongsan, South Korea) approved protocols and guidelines for this study (7002016-A-2014-005).

Plasma analysis

Blood was obtained following overnight fasting from NS-F, NS-M, and S-M subjects. Blood was collected using a vacutainer (BD Biosciences, Franklin Lakes, NJ, USA) containing EDTA (final concentration, 1 mM) at 0, 4, and 8 weeks during intake of Vit C. Plasma was isolated by low-speed centrifugation (3,000 rpm) and stored at -80˚C until analysis. To analyze plasma, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured using commercially available kits (Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). Vitamin C level in plasma of each subject was measured in according to previous method described by Aye (24).

Characterization of lipoproteins

Very low-density lipoprotein (VLDL, d <1.019 g/mL), LDL (1.019 <d < 1.063), HDL_2 (1.063 < d < 1.125), and HDL_3 (1.125 < d < 1.225) were isolated from pooled NS-F, NS-M, and S-M plasma subjects via sequential ultracentrifugation (25), with the density adjusted by addition of NaCl and NaBr in accordance with standard protocols. Samples were centrifuged for 22 hours at 10 °C at 100,000 g using a Himac CP-100WX (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center of Yeungnam University. To analyze lipoproteins, total cholesterol (TC) and triglyceride (TG) levels were measured using commercially available kits (Cleantech TS-S; Wako Pure Chemical, Osaka, Japan). The protein concentrations of lipoproteins were determined via Lowry protein assay, as modified by Markwell et al. (26). Expressional levels of apoA-I and apo-B were determined by Western blotting.

To assess the degree of lipoprotein oxidation, the concentration of oxidized species in lipoprotein was determined by thiobarbituric acid-reacting substance (TBARS) assay method using malondialdehyde as a standard (27). To compare the extent of glycation between groups, advanced glycation end products (AGEs) in lipoproteins were determined from reading the fluorometric intensities at 370 nm (excitation) and 440 nm (emission), as described previously (28) using a spectrofluorometer LS55 (Perkin Elmer, Shelton, CT, USA) with the WinLab software package (version 4.0).

Western blot

Apolipoprotein/lipoprotein compositions were compared via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using identical protein loading amounts of LDL and HDL_3 (6 μg of total protein per lane), and the expression level of apolipoprotein was measured via immunodetection. Goat anti-human apoB antibody (ab742) was purchased

from Chemicon (Billerica, MA, USA) and rabbit anti-human apoA-I antibody [EP1368Y] (ab52945) was purchased from Abcam (Cambridge, UK) as the primary antibody. Anti-goat immunoglobulin G (SC2020; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-rabbit immunoglobulin G (SC2004; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as the secondary antibody. The relative band intensity (BI) was compared via band scanning with Gel Doc® XR (Bio-Rad) using Quantity One software (version 4.5.2).

LDL phagocytosis assay

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection (ATCC, #TIB-202™, Manassas, VA, USA) and maintained in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum until needed. Cells that had undergone no more than 20 passages were incubated in medium containing phorbol 12-myristate 13-acetate (PMA, 150 nM) in 24-well plates for 48 hours at 37°C in a humidified incubator (5% CO_2, 95% air) in order to induce differentiation into macrophages. Differentiated and adherent macrophages were then rinsed with warm PBS, followed by incubation with 450 μL of fresh RPMI-1640 medium containing 0.1% FBS and 50 μg of each LDL (1 mg of protein/mL in PBS) for 48 hours at 37°C in a humidified incubator. After incubation, cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 10 minutes. Next, fixed cells were stained with oil-red O staining solution (0.67%) and washed with distilled water. THP-1 macrophage-derived foam cells were then observed and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 400x magnification.

149 ***Anti-atherosclerotic assay***

150 Oxidized LDL (oxLDL) was produced by incubating the LDL fraction with $CuSO_4$
151 (final concentration: 10 μM) for 4 hours at 37°C. The oxLDL was then filtered through a
152 0.22-μm filter (Millex; Millipore, Bedford, MA) and analyzed by thiobarbituric acid–reacting
153 substances (TBARS) assay to determine the extent of oxidation as described previously (27).
154 The differentiated and adherent macrophages were then rinsed with warm PBS and incubated
155 with 400 μL of fresh RPMI-1640 medium containing 0.1% fetal bovine serum, 50 μg of
156 oxLDL (1 mg of protein/mL in PBS), and 30 μg of each HDL_2 (1 mg of protein/mL in PBS)
157 or HDL_3 (2 mg of protein/mL in PBS) for 48 hours at 37°C in a humidified incubator. After
158 incubation, cells were stained with oil-red O solution (0.67%) to visualize the amount of lipid
159 species in cells. THP-1 macrophage-derived foam cells were then observed and photographed
160 using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 400x magnification.
161 Quantification of stained area was carried out via computer-assisted morphometry
162 using Image Proplus software (version 4.5.1.22, Media Cybernetics, Bethesda, MD).
163
164 ***Anti-senescence assays***
165 In order to test anti-senescence activity of HDL from each group after vit C
166 consumption, the HDL from each group was treated into human dermal fibroblasts (HDFs).
167 Because aging stress such as glycation caused cellular senescence as our previous report (15),
168 there is a possibility that functionally enhanced HDL can inhibit the senescence. Primary
169 HDFs were cultured in Dulbecco′s modified Eagle′s medium (DMEM; Life Technologies;
170 Gaithersburg, MD, USA). HDFs were plated in DMEM at a density of 2×10^4 cells per well

171 in a 6-well plate and cultured at 37°C in a humidified incubator. HDFs were exposed at
172 passage 6 (approximately 40% confluence) to the indicated concentrations of 30 μg each of
173 HDL_2 or HDL_3 for 30 days with sub-culture to passage 15.

174 Extent of aging and cellular SA-β-gal activity was compared as previously described.
175 Cells were fixed for 5 minutes in 4% paraformaldehyde in PBS, washed three times in PBS,
176 and incubated in SA-β-gal staining solution (40 mM citric acid/phosphate [pH 6.0], 5 mM
177 potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, and 1
178 mg/mL of 5-bromo-4-chloro-3-indolyl-X-galactosidase) for 16 hours at 37°C. SA-β-gal−
179 stained HDF cells were then observed and photographed using a Nikon Eclipse TE2000
180 microscope (Tokyo, Japan) at 400x magnification (29).
181

182 ***Measurement of facial skin melanin content***

183 During Vit C supplementation, facial skin conditions of all participants were measured
184 every week using a multi-probe adapter system (MPA5, Courage+Khazaka electronic,
185 Cologne, Germany). Probes for facial melanin and erythema were measured using a
186 mexameter (MX18, Courage+Khazaka electronic, Cologne, Germany). Moisture and sebum
187 in facial skin were measured by a corneometer (CM825) and sebumeter (SM815),
188 respectively, from the Courage+Khazaka Electronics. In order to minimize bias, we measured
189 the same position of the cheekbone three times and averaged the data from subjects under the
190 same room temperature and humidity.
191

192 ***RNA isolation and real-time PCR***

193 Total RNA was isolated from 200 μL of purified HDL_3 (3.5 mg/mL, total 700 μg of
194 HDL_3) using TRIzol reagent and a miRNeasy easy Kit (Qiagen, Hilden, Germany) as
195 previously reported (30). For normalization of miR expression, 5 nmol/L of *C. elegans* miR-
196 39 (RNA oligonucleotide, Sigma-Aldrich) was added during RNA extraction. Purified RNA
197 (10 μL) from purified HDL (24 μL) was used for reverse transcription reaction in a total
198 volume of 20 μL using a miScript PCR starter kit (Qiagen, Hilden, Germany). Real-time PCR
199 for detection of mature miRNA was performed using a miScript SYBR Green PCR Kit
200 (Qiagen, Hilden, Germany). To quantify miRNA155 and *C. elegans* miR-39, we used the
201 following primer sets: miRNA155, ttaatgctaatcgtgataggggt; *C. elegans* miR-39,
202 tcaccgggtgtaaatcagcttg.
203
204 ***Data analysis***

205 All data are expressed as the mean ± SD from at least three independent experiments
206 with duplicate samples. Data comparisons were assessed by the Student's *t*-test using the
207 SPSS program (version 14.0; SPSS, Inc., Chicago, IL, USA). In the human study, data in the
208 same group were evaluated via one-way analysis of variance (ANOVA) using SPSS (version
209 14.0; Chicago, IL, USA), and the differences between the means were assessed using
210 Duncan′s multiple-range test. Statistical significance was defined as a $p<0.05$.
211
212
213

RESULTS

Plasma parameters

All participants consumed Vit C for 8 weeks and tolerated well the Vit C supplementation without notable side effects. As shown in Table 1, from weeks 0 to 8, all groups showed no significant changes in anthropometric data, including body weight, serum cholesterol, and TG level. Interestingly, among the groups, S-M group showed around 1.6-fold higher TG levels than NS-M group over 8 weeks. However, serum HDL-C level was significantly elevated in all groups after 8 weeks of the Vit C consumption. NS-F group showed an 8% increase by week 8 (65±18 mg/dL) from week 0 (60±13 mg/dL). NS-M group showed significant elevation of HDL-C level up to 10% by week 8. Whereas non-smoking (NS-F and NS-M) group showed no elevation of HDL-C by week 4, S-M group showed a 10% increase in HDL-C from initial level. By week 8, S-M group showed the most notable increase in HDL-C level (16%) from week 0. All groups showed similar levels of serum glucose between 80-90 mg/dL during the 8 weeks.

Serum antioxidant abilities based on FRAP determination were enhanced in a time-dependent manner in all groups, as shown in Fig. 1A. FRAP increased up to 45% and 30% in the NS-F and NS-M groups, respectively, 8 weeks. Smoker group showed 10% and 30% increases in FRAP by weeks 4 and 8, respectively.

In female group, serum level of vitamin C was elevated upto 2-fold higher than initial level as shown in Table 1. In male, non-smoker and smoker group showed 1.4-fold and 1.8-fold elevation of vit C level than initial level by 8 week consumption (Table 1).

Extent of glycation in lipoproteins was reduced after vitamin C intake

All groups showed significant and reduction of AGE content in lipoproteins following Vit C consumption (Fig. 1B) based on fluorescence determination. In LDL fractions, NS-F group showed the highest reduction of AGE content up to 40%, whereas NS-M group showed a 19% reduction. In HDL_2 fractions, NS-M group showed the largest reduction of AGE content up to 20%, whereas S-M group showed a 15% decrease. These results indicate that glycation of lipoproteins can be prevented by consumption of Vit C in a time-dependent manner.

Lipoprotein properties and compositional change

In LDL fraction, as shown in Suppl. Table 1, male groups showed diminished TC and TG contents during 8 weeks with the same protein content, whereas females showed decreased TC content only. In NS-M group, TC and TG contents were reduced by 23% and 27% from initial levels, respectively. S-M group showed 5% and 7% reduced TC and TG contents in LDL. This result suggests that Vit C supplementation can improve lipid composition of LDL, especially in male non-smoker subjects. Furthermore, reduced TG content in lipoproteins suggests that better quality LDL can reduce atherogenic and inflammation.

In HDL_2 fraction, all groups showed elevated TC content, whereas TG content was unchanged. NS-F and S-M groups showed 46% and 11% increased TC contents, respectively (Suppl. Table 1). Interestingly, NS-M group showed significant elevation of TC content in HDL_3 fraction up to 56%, whereas there was no TC elevation in HDL_2. On the other hand, NS-F group showed no elevation of TC in HDL_3. All groups showed reduced TG content in HDL_3 during 8 weeks supplementation of Vit C.

Expression of apolipoproteins

At week 0, all groups showed a distinct apo-B_{48} band (Fig. 2A), although it disappeared by weeks 4 to 8. Immunodetection also revealed that the apoB-48 band disappeared, especially for male subjects (Fig. 2B). NS-M and S-M groups showed 92% and 83% reduction of apo-B_{48} band intensity, respectively, whereas NS-F group showed a 20% reduction (Fig. 2B). On the other hand, apoA-I band intensity in HDL was elevated by week 8 in all groups. As shown in Fig. 2C, apoA-I band intensity in HDL_2 increased up to 21% in the NS-F group, whereas NS-M and S-M groups showed a 13% increase. Interestingly, S-M group showed significant elevation of apoA-I band intensity in HDL_3 up to 22% after 8 weeks of Vit C consumption (Fig. 2D). These results suggest that composition and expressional levels of apolipoproteins in lipoproteins were notably influenced by the Vit C consumption.

Uptake of LDL into macrophages was inhibited by the vit C consumption

Phagocytosis of modified LDL, such as oxidation and glycation, into macrophages is a well known atherosclerotic process to produce foam cell. More oxidized LDL or glycated LDL is more retained in the cell to initiate atherogenic process. Individually purified LDL from each group was applied to THP-1 cells in order to test extent of phagocytosis, as in our previous report (13). At week 0, as shown in Fig. 3, LDL-treated cells from S-M group showed the strongest red intensity (14.3- and 4.2-fold greater compared to NS-F and NS-M groups, respectively), suggesting that LDL from S-M group was more easily taken up into macrophages to initiate atherogenesis. By week 8, S-M LDL-treated cells showed much less

red intensity (86% reduction as compared to other group), suggesting that the atherogenic properties of LDL were improved by Vit C as shown in Fig. 3A and 3B ($p<0.001$).

Anti-atherogenic functions of HDL were enhanced by the vit C

Generally, healthy HDL exerts anti-atherogenic activity via inhibition of LDL oxidation and oxLDL phagocytosis into macrophage. As shown in Suppl. Fig. 2A, HDL from non-smokers inhibited phagocytosis of oxLDL, and HDL from all subjects at 8 weeks showed enhanced anti-atherogenic activities. In HDL_2 fraction at week 0, S-M HDL_2-treated cells showed the strongest red intensity, although it was 22% less than oxLDL alone. However, HDL-treated cells at week 8 showed 40%, 49%, and 61% reduction of red intensity for NS-F, NS-M, and S-M groups, respectively.

S-M HDL_2-treated cells at week 0 showed the strongest red intensity (1.3- and 1.4-fold greater compared to NS-F and NS-M groups, respectively), suggesting that HDL_3 was severely modified by smoking, as in our previous report (13). However, red intensity was diminished by 57%, 49%, and 64% in the NS-F, NS-M, and S-M groups, respectively, by 8 weeks (Suppl. Fig. 2B). These results suggest that 8 weeks of Vit C consumption improved functionality of HDL, especially in terms of anti-atherosclerotic activity.

Cellular senescence was inhibited by the HDL in human dermal cells

As shown in Suppl. Fig. 3A, at week 0, HDL_2 from S-M group resulted in greater senescence than that from NS-F or NS-M group (11% greater than PBS alone), whereas NS-F and NS-M groups showed 6% and 4% less senescence than PBS alone, respectively. However,

after 8 weeks of consumption, senescence was improved by HDL_2 as visualized by SA-β-gal staining, with 33%, 26%, and 26% reduction of NS-F, NS-M, and S-M groups, respectively.

At week 0, all HDL_3 fractions showed enhanced anti-senescence activity compared to HDL_2, suggesting that HDL_3 has greater anti-aging activity than HDL_2. After 8 weeks, all groups showed less cellular aging, with 19%, 12%, and 13% reduction of SA-β-gal staining in NS-F, NS-M, and S-M groups, respectively (Suppl. Fig. 3B).

Melanin contents in facial skin are diminished by Vit C consumption

As shown in Suppl. Fig. 4, at week 0, S-M group showed the highest melanin content in facial skin at the cheekbone (44% and 17% darker than NS-F and NS-M groups, respectively). After 8 weeks of Vit C consumption, melanin content of S-M group was significantly reduced by 21% ($p<0.05$), whereas NS-M and NS-F groups showed 5% reduction over the same period. S-M group showed similar melanin content in facial skin as the NS-M group after a high dose of Vit C. These results suggest that Vit C supplementation can improve facial skin whitening, especially in smokers group.

Change in miR155 expression in HDL by vitamin C

In LDL fraction, there was no significant amount of miR155 and no difference between groups during the entire period. However, miR155 was abundant in HDL fraction and was elevated in S-M group at week 0, showing 3- and 2-fold higher levels in HDL_2 and HDL_3 fractions than NS-M group (Fig. 4). This result suggests that miR155 expression was elevated by smoking. After 8 weeks of Vit C consumption, NS-M and S-M groups showed 93% and

92% reduction of miR155 level in HDL_3 fraction, respectively, suggesting that a high dose of Vit C can improve anti-inflammatory function via reduction of inflammatory miRNA.

DISCUSSION

Although the beneficial functions of Vit C are well known, the mechanism of its effects on lipoprotein metabolism remains to be investigated. Therefore, we analyzed serum lipoproteins from normal subjects and smokers who consumed 1,250 mg of Vit C for 8 weeks. After quantification of vit C level, we found that all subjects showed significant elevation of ferric ion reduction ability and serum vit C level after 8 weeks consumption (Table 1). This result makes a good correlation with increase of antioxidant and anti-glycation ability in lipoprotein fractions. However, the increase extent of vit C level in serum was relatively small compared with the high dose of consumption although the serum contained EDTA and stored in -80 ˚C until analysis, which can cause bias for colorimetric measurement.

Interesting finding of the current study is apo-B_{48} in LDL was diminished while apo-B_{100} was elevated by Vit C supplementation (Fig. 2), although there were no notable difference in serum lipids (cholesterol and triacylglycerol) and glucose levels among the groups. Apo-B_{48} is a constituent of chylomicron remnants and TG-rich lipoproteins, and its high level is suspected to be a major risk factor for the development of cardiovascular disease (31). In our results, TG content in each lipoprotein fraction, including LDL, was significantly reduced in all groups following Vit C consumption, suggesting that the properties of LDL became less atherogenic.

Our group recently reported that smokers show higher serum TG levels, and lipoproteins from smokers are strikingly enriched with TG (13). The current finding of elevated TG content in lipoproteins from smokers at week 0 (Suppl. Table 1) is in good agreement with the report (13). In addition to lipid composition, extent of glycation and oxidation of lipoproteins is increased by smoking and decreases particle size.

MicroRNAs represent a class of small non-coding RNAs of 22 nucleotides in length that post-transcriptionally modulate the expression of target genes and are thus involved in many cellular processes, including cell proliferation, apoptosis, and inflammation (33). Recently, miRNAs have been identified as potential modulators of the immune response (34). In studies in miR-155 knockout mice, miR-155 was found to be essential for development of immune cells (35) and T cell differentiation (36). HDL from CAD patients has totally different miRNA profile compared to that of normal subjects. Among them, miR155 is a well known regulator of inflammation and is up-regulated in patients with CAD (37) as well as aortas of apoE-/- mice fed a high-fat diet for 3-10 months (38), suggesting its role as an inflammatory marker. Inflammation and macrophage activation are mediated by ROS, which are counteracted by antioxidants. MiR155 expression was highly induced by toll-like receptor (TLR) agonist activation of macrophage. Specific TLR-4 ligands such as lipopolysaccaride (LPS) or oxLDL induced the expression of miR155 in primary mouse macrophages (39). As a dietary antioxidant, α-tocopherol regulates inflammatory miRNA gene expression in macrophages (40) and animals (41). Taken together with our current results, the inhibition of oxLDL production and phagocytosis into macrophage (Fig. 2 and Suppl. Fig. 2) were correlated well with lowering of miR155 level in HDL (Fig. 4) in smokers group.

To the best of our knowledge, this is the first report to investigate the vit C consumption can modulate miRNA content in lipoproteins. In this study, we measured the miR155 level in lipoproteins purified from smokers and non-smokers after Vit C intake (Fig. 4). Interestingly, the miR155 level was significantly reduced especially in HDL by the vit C consumption, suggesting that circulating miRNA content in HDL can be altered by the nutritional treatment, as well as compositions of apolipoproteins and lipids. Major finding of this study is that

impaired functionality of the smoker's HDL can be enhanced more by the high-dose vit C consumption for 8 weeks.

Although it has been well known that vitamin C is widely used for the treatment of melasma and chloasma because of its inhibitory action on melanogenesis (42). The current result suggests that high dosage consumption of vit C during 8-weeks resulted facial skin whitening in young smokers (Suppl. Fig. 4), via inhibition of dermal cell senescence and anti-oxidant activity by enhanced HDL (Suppl. Fig. 3). Further study should be investigated to elucidate detail mechanism, this result might be associated with the enhanced function of HDL; anti-oxidant (Fig. 1), anti-glycation (Fig. 3), anti-senescence (Suppl. Fig. 3), anti-inflammatory activity (Fig. 3 and 4).

In conclusion, in this study, we characterized functions and properties of HDL and its content of miR155 expression from smokers and non-smokers after the Vit C consumption. Daily consumption of a high dose of vitamin C for 8 weeks resulted in enhanced anti-senescence and anti-atherosclerotic effects via improvement of lipoprotein parameters and microRNA expression through anti-oxidation and anti-glycation.

Conflict of interest

All authors declare no conflict of interest.

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398 Planning of Korea.

REFERENCES

1. O. Satoshi, O. Yumiko, S. Nobutaka, S. Gen-ichiro and I. Masaki, *Anticancer Res.*, 2009, **29**, 809-816.
2. R. M. Salonen, K. Nyyssönen, J. Kaikkonen, E. Porkkala-Sarataho, S. Voutilainen, T. H. Rissanen, T. P. Tuomainen, V. P. Valkonen, U. Ristonmaa, H. M. Lakka, M. Vanharanta, J. T. Salonen and H. E. Poulsen, *Circulation*, 2003, **107**, 947-953.
3. C. K. Ferrari and E. A. Torres, *Biomed. Pharmacother.*, 2003, **57**, 251-260.
4. G. Nikola, *Radiat. Phys. Chem.*, 2007, **76**, 577–586.
5. M. H. Park, D. H. Kim, E. K. Lee, N. D. Kim, D. S. Im, J. Lee, B. P. Yu and H. Y. Chung, *Arch. Pharm. Res.*, 2014, **37**, 1507-1514
6. D. S. Celermajer, K. E. Sorensen, D. J. Spiegelhalter, D. Georgakopoulos, J. Robinson and J. E. Deanfield, *J. Am. Coll. Cardiol.*, 1994, **24**, 471-476.
7. W. Michael, *Arterioscler. Thromb. Vasc. Biol.*, 2009, **29**, 1244-1250.
8. B. Christie, A. Bruce and S. James, *Eur. Heart J.*, 2005, **26**, 2224–2231.
9. J. B. Echouffo-Tcheugui and A. P. Kengne, *Diabetes Metab.*, 2013, **39**, 389-396.
10. K. H. Cho, *BMB Rep.*, 2009, **42**, 393-400.
11. G. Ferretti, T. Bacchetti, A. Nègre-Salvayre, R. Salvayre, N. Dousset and G. Curatola, *Atherosclerosis*, 2006, **184**, 1-7.
12. C. C. Hedrick, S. R. Thorpe, M. X. Fu, C. M. Harper, J. Yoo, S. M. Kim, H. Wong and A. L. Peters, *Diabetologia*, 2000, **43**, 312-320.
13. K. H. Park, D. G. Shin and K. H. Cho, *Toxicol. Sci.*, 2014, **140**, 16–25.
14. K. H. Park, D. G. Shin, J. R. Kim and K. H. Cho, *J. Gerontol. A Biol. Sci. Med. Sci.*, 2010, **65**, 600-610.

15. K. H. Park and K. H. Cho, *J. Gerontol. A Biol. Sci. Med. Sci.*, 2011, **66**, 511-520.

16. K. H. Cho, S. H. Park, J. E. Park, Y. O. Kim, I. Choi, J. J. Kim and J. R. Kim, *Clin. Biochem.*, 2008, **41**, 56-64.

17. H. Lee, J. E. Park, I. Choi and K. H. Cho, *BMB Rep.*, 2009, **42**, 605-610.

18. K. H. Park, J. M. Kim and K. H. Cho, *Mol. Nutr. Food Res.*, 2014, **58**, 1537-1545.

19. Q. Chen, M. G. Espey, A. Y. Sun, C. Pooput, K. L. Kirk, M. C. Krishna, D. B. Khosh, J. Drisko and M. Levine, *Proc. Natl. Acad. Sci. USA.*, 2008, **105**, 11105-11109.

20. K. C. Vickers, B. T. Palmisano, B. M. Shoucri, R. D. Shamburek and A. T. Remaley, *Nat. Cell Biol.*, 2011, **13**, 423-433.

21. P. S. Mitchell, R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari, *Proc. Natl. Acad. Sci. USA.*, 2008, **105**, 10513-10518.

22. E. E. Creemers, A. J. Tijsen and Y. M. Pinto, *Circ. Res.*, 2012, **110**, 483-495.

23. R. L. Eigsti, B. Sudan, M. E. Wilson and J. W. Graff, *J. Biol. Chem.*, 2014, **289**, 28433-28447.

24. K. Aye, *Clin. Chim. Acta.*, 1978, **86**, 153-157.

25. R. J. Havel, H. A. Eder and J. H. Bragdon, *J. Clin. Invest.*, 1955, **34**, 1345-1353.

26. M. A. Markwell, S. M. Haas, L. L. Bieber and N. E. Tolbert, *Anal. Biochem.*, 1978, **87**, 206-210.

27. M. S. Blois, *Nature*, 1958, **191**, 1199-1200.

28. J. D. McPherson, B. H. Shilton and D. J. Walton, *Biochemistry*, 1988, **27**, 1901-1907.

29. K. H. Cho, *J. Gerontol. A Biol. Sci. Med. Sci.*, 2011, **66**, 1274-1285.

30. J. Wagner, M. Riwanto, C. Besler, A. Knau, S. Fichtlscherer, T. Röxe, A. M. Zeiher, U. Landmesser and S. Dimmeler, *Arterioscler. Thromb. Vasc. Biol.*, 2013, **33**, 1392-1400.

31. K. G. Jackson and C. M. Williams, *Atherosclerosis*, 2004, **176**, 207-217.

32. A. Patel, F. Barzi, K. Jamrozik, T. H. Lam, H. Ueshima, G. Whitlock and M. Woodward, *Circulation*, 2004, **110**, 2678-2686.

33. C. Urbich, A. Kuehbacher and S. Dimmeler, *Cardiovasc. Res.*, 2008, **79**, 581-588.

34. R. M. O'Connell, D. S. Rao, A. A. Chaudhuri and D. Baltimore, *Nat. Rev. Immunol.*, 2010, **10**, 111-122.

35. A. Rodriguez, E. Vigorito, S. Clare, M. V. Warren, P. Couttet, D. R. Soond, S. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, D. Vetrie, K. Okkenhaug, A. J. Enright, G. Dougan, M. Turner and A. Bradley, *Science*, 2007, **316**, 608-611.

36. T. H. Thai, D. P. Calado, S. Casola, K. M. Ansel, C. Xiao, Y. Xue, A. Murphy, D. Frendewey, D. Valenzuela, J. L. Kutok, M. Schmidt-Supprian, N. Rajewsky, G. Yancopoulos, A. Rao and K. Rajewsky, *Science*, 2007, **316**, 604-608.

37. J. Zhu, T. Chen, L. Yang, Z. Li, M. M. Wong, X. Zheng, X. Pan, L. Zhang and H. Yan, *PLoS One*, 2012, **7**, e46551.

38. M. Nazari-Jahantigh, Y. Wei, H. Noels, S. Akhtar, Z. Zhou, R. R. Koenen, K. Heyll, F. Gremse, F. Kiessling, J. Grommes, C. Weber and A. Schober, *J. Clin. Invest.*, 2012, **122**, 4190-4202.

39. K.D.Taganov, M.P. Boldin, K. J. Chang, D. Baltimore, *Proc Natl Acad Sci U S A* 2006, **103**, 12481-12486

40. C. Boesch-Saadatmandi, A. Loboda, A. E. Wagner, A. Stachurska, A. Jozkowicz, J. Dulak, F. Döring, S. Wolffram and G. Rimbach, *J. Nutr. Biochem.*, 2011, **22**, 293-299.

41. S. Gaedicke, X. Zhang, C. Schmelzer, Y. Lou, F. Doering, J. Frank and G. Rimbach, *FEBS Lett.*, 2008, **582**, 3542-3546.

42. A. B. Lerner and T. B. Fitzpatrick, *Physiol. Rev.*, 1950, **30**, 91–126.

Figure legends

FIGURE 1. Change of antioxidant ability and vit C level in serum during 8 weeks consumption of vit C.

A. Ferric ion reduction ability of serum after vitamin C consumption using ferric ion reducing ability of plasma (FRAP). * and ** indicates $p<0.05$ and $p<0.01$ versus 0 week in the SM group, respectively; ## and §§ indicates $p<0.01$ versus 0 week in the NS-F and NS-M group, respectively.

B. Quantification of advanced glycated end products in LDL, HDL_2, and HDL_3 by fluorospectrometric measurement (Ex=370 nm, Em=440 nm).

FIGURE 2. Expression level of apoB in LDL and apoA-I in HDL after consumption of Vit C. Number indicates designated week. BI, band intensity.

A. Electrophoretic patterns of apo-B_{100} and Apo-B_{48} in LDL.

B. Immunodetection of apo-B_{100} and Apo-B_{48} in LDL.

C. Immunodetection of apolipoprotein A-I (apoA-I) in each HDL_2 (6 μg/lane).

D. Immunodetection of apolipoprotein A-I (apoA-I) in each HDL_3 (6 μg/lane)

FIGURE 3. LDL uptake into macrophages with purified LDL from each subject after 0 and 8 weeks of Vit C consumption. Extent of oxLDL phagocytosis are visualized by oil-red O staining (A) and the stained area was quantified by image analysis software (B).

FIGURE 4. Expressional level of microRNA in HDL_3 purified after consumption of Vit C.

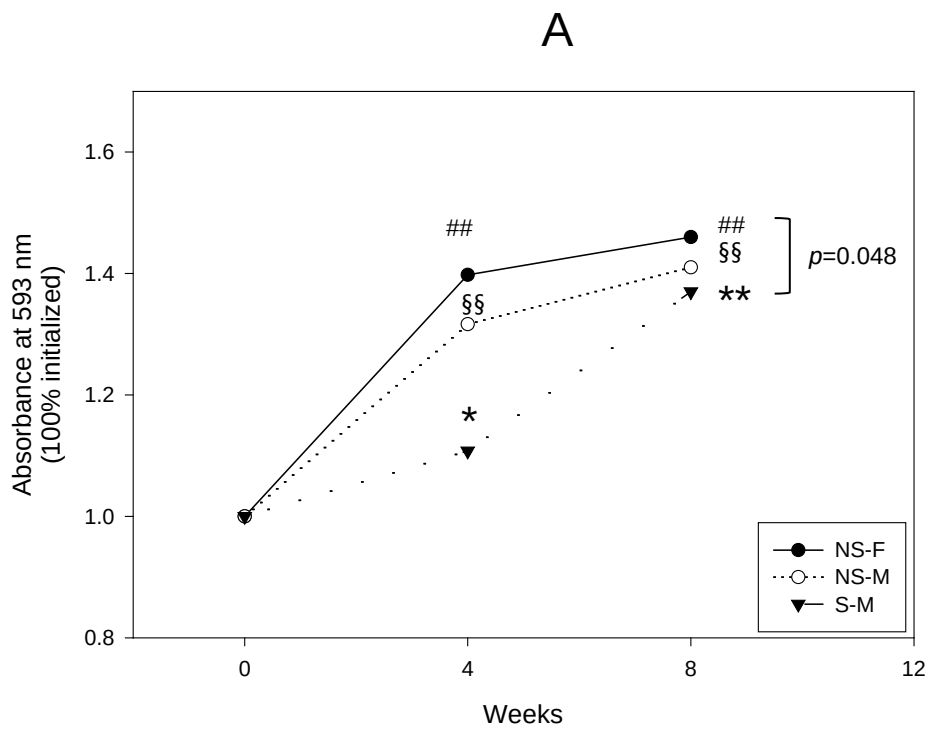
TABLE 1. Change of plasma profiles during vitamin C supplementation during 8 weeks (mean ± SD).

	NS-F (n=14)			NS-M (n=20)			S-M (n=12)		
	0w	4w	8w	0w	4w	8w	0w	4w	8w
Age (years)		20.3±1.0			21.3±1.5			24.0±1.7	
BMI (kg/m^2)	21.2±2.6	21.1±2.8	21.1±2.7	22.9±2.1	23.1±2.1	23.5±2.3	23.5±1.8	23.4±1.6	23.7±1.8
TC (mg/dL)	166±18	167±26	170±34	165±32	162±35	169±25	175±33	170±37	180±34
TG (mg/dL)	64±15	70±33	60±16	78±27	74±45	80±47	125±62	146±61	126±58
HDL-C (mg/dL)	60±13^{a}	59±16^{a}	65±18$^{b, *}$	57±6^{a}	57±7^{a}	63±6$^{b, *}$	50±4^{a}	55±4^{ab}	58±3$^{b, *}$
LDL-C (mg/dL)[1]	94±12	94±20	94±23	93±31	90±31	90±23	101±19	99±22	96±26
Glucose (mg/dL)	79±21	87±5	78±7	83±10	79±15	80±7	91±4	85±6	86±8
AST (IU/L)	11±1	10±1	9±2	8±1	9±2	10±2	10±2	9±4	9±2
ALT (IU/L)	5±1	6±1	5±1	6±2	6±2	8±2	6±1	6±1	8±1
Vitamin C (μM)	9±4^{a}	11±3^{a}	18±3$^{b, **}$	16±4^{a}	19±5^{a}	23±2$^{b, *}$	17±3^{a}	19±5^{a}	32±4$^{b, **}$

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol, TC, total cholesterol; TG, triglyceride; NS-F, non-smoker female; NS-M, non-smoker male; S-M, smoker male

$^{*}p<0.05$; **, $p<0.01$ versus 0-week in each group; [1], calculated LDL cholesterol using Friedewald equation (LDL-C=TC-HDL-C-TG/5).

[a] The mean values not sharing a common letter in the same row are significantly different in the same groups ($p < 0.05$).



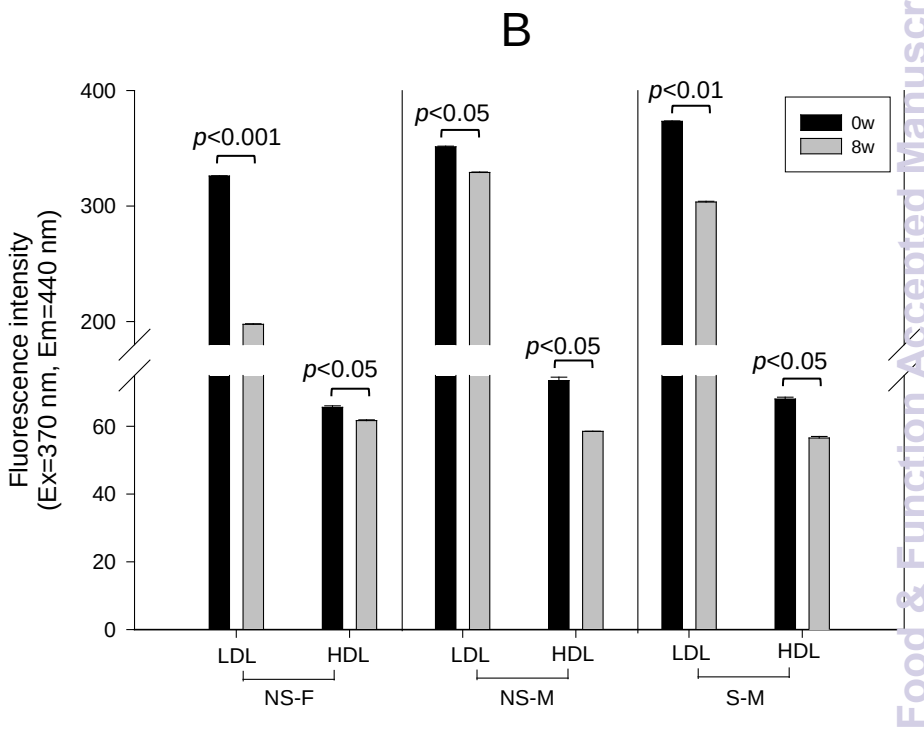
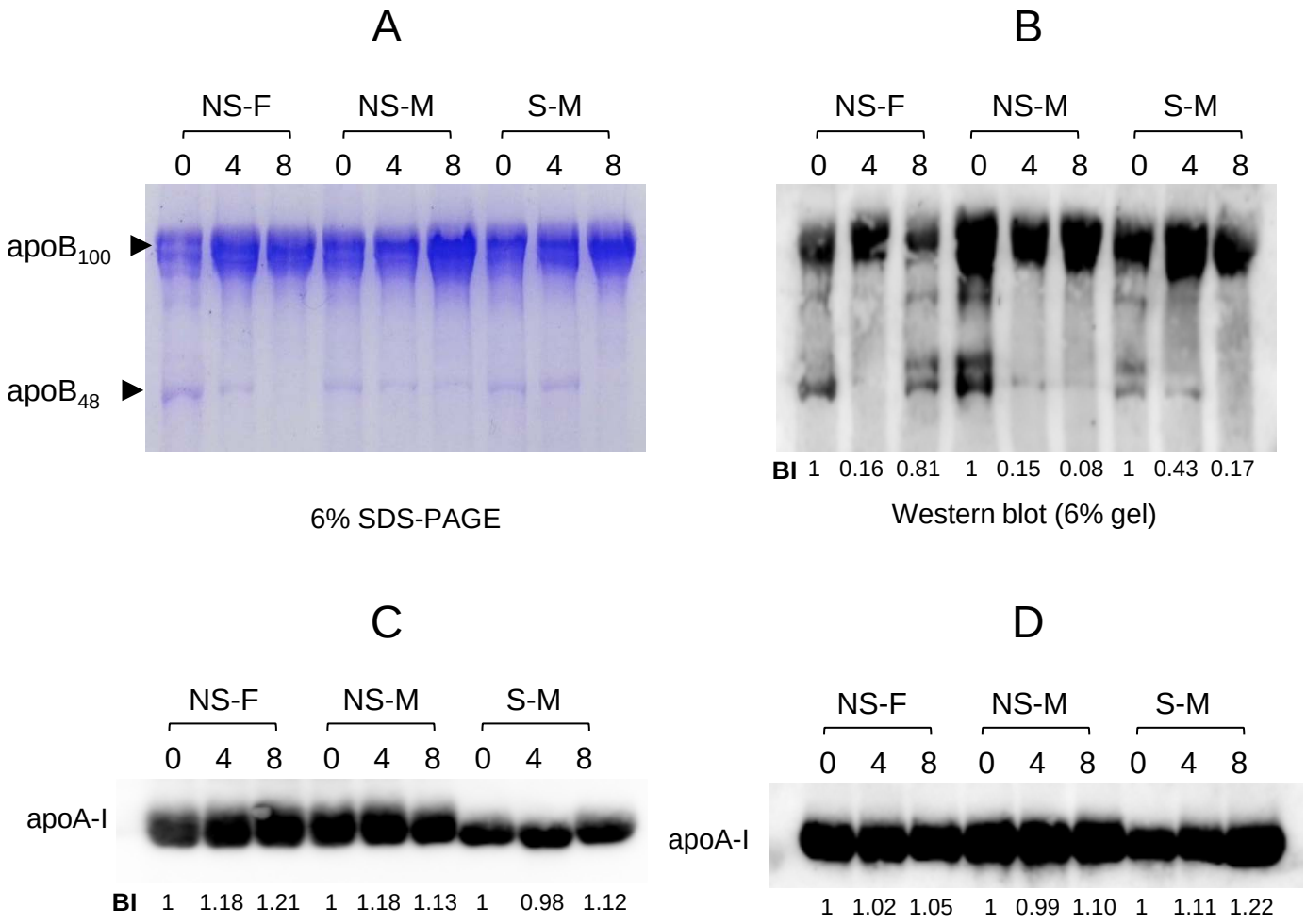


Fig. 2



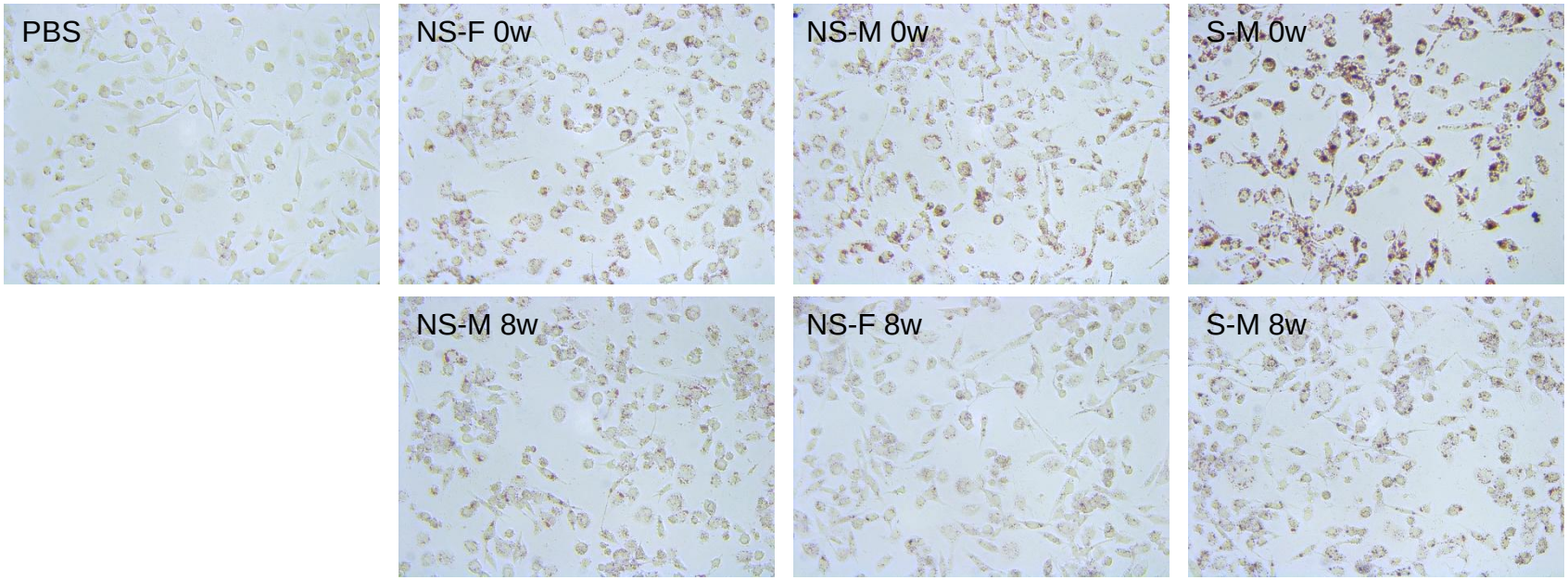
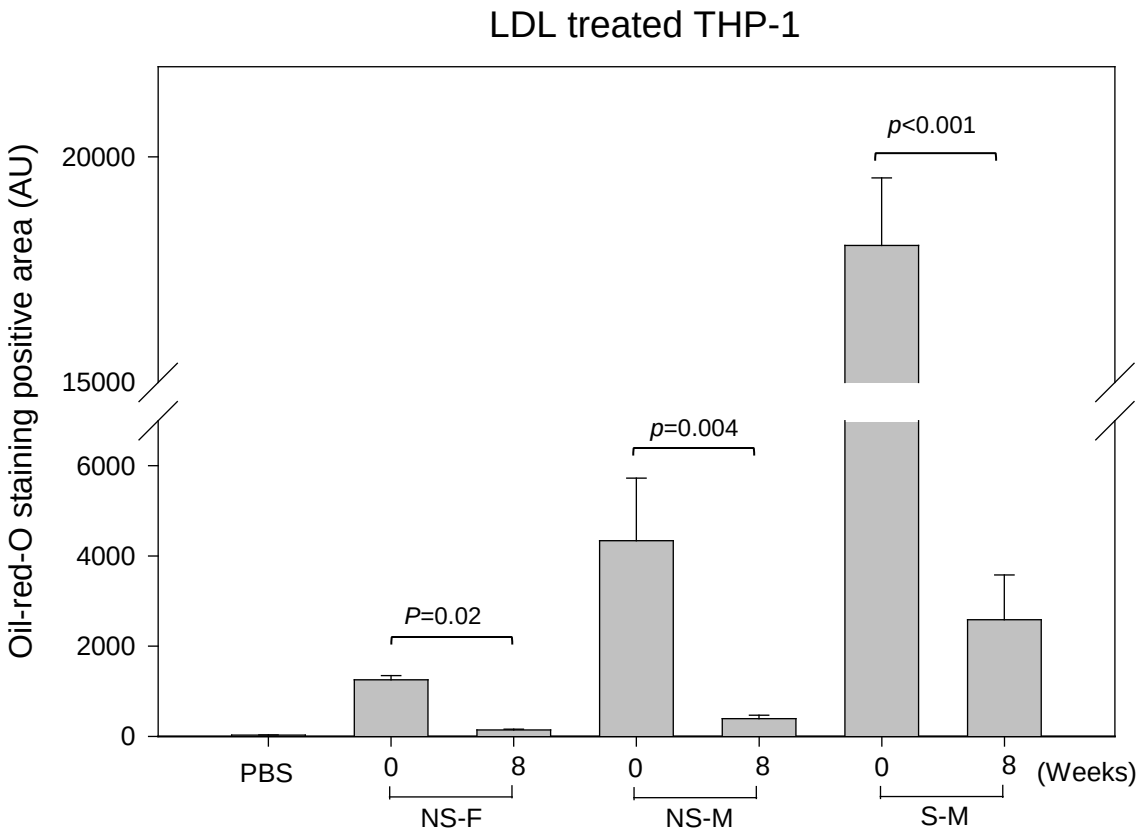
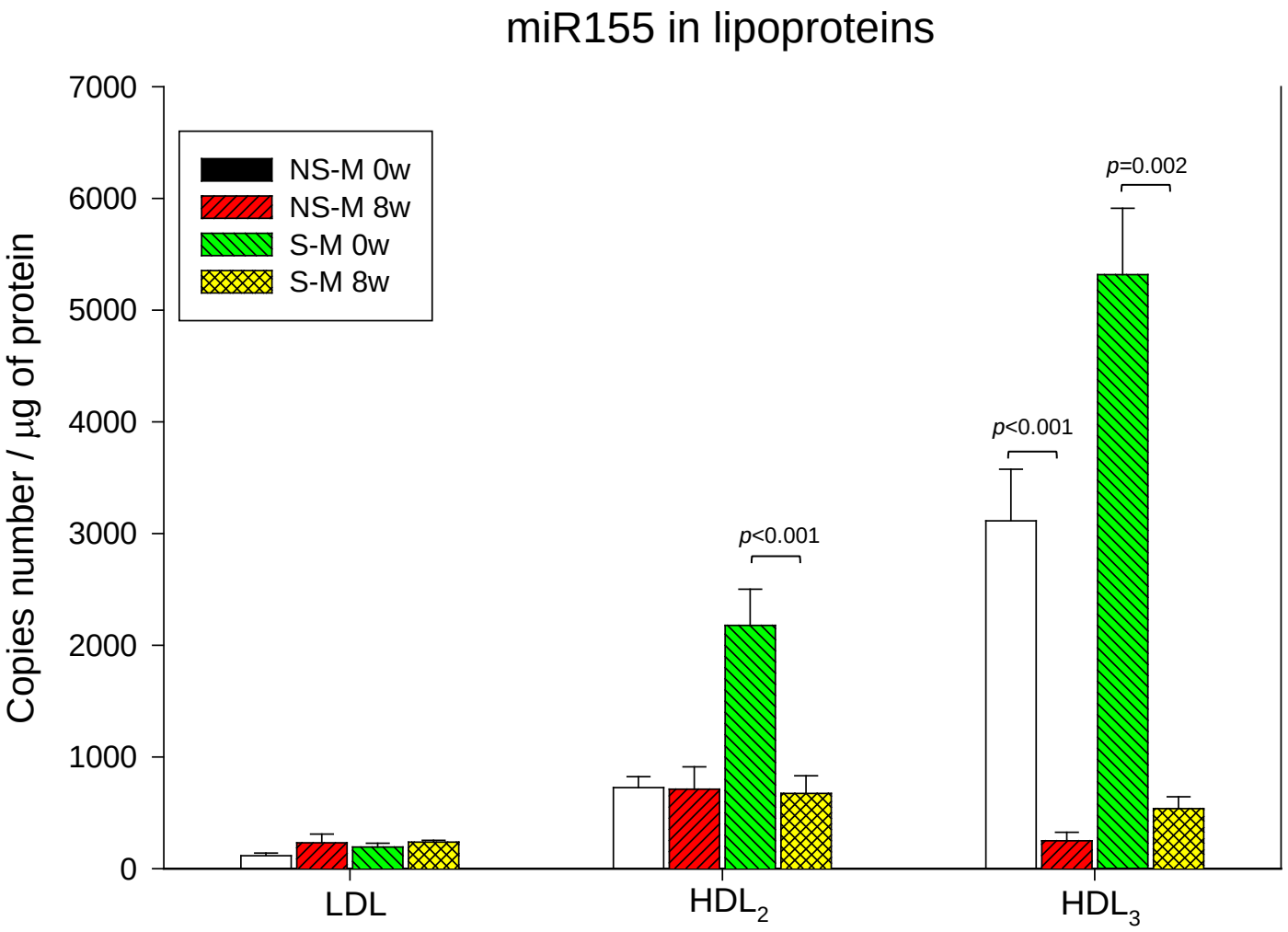
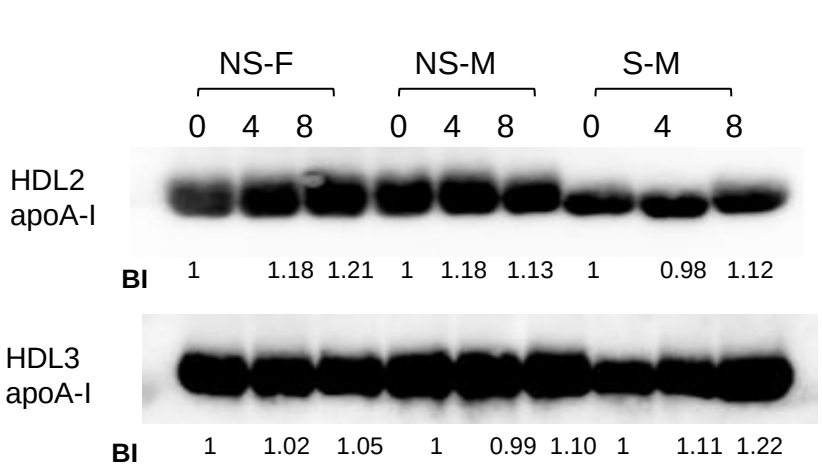


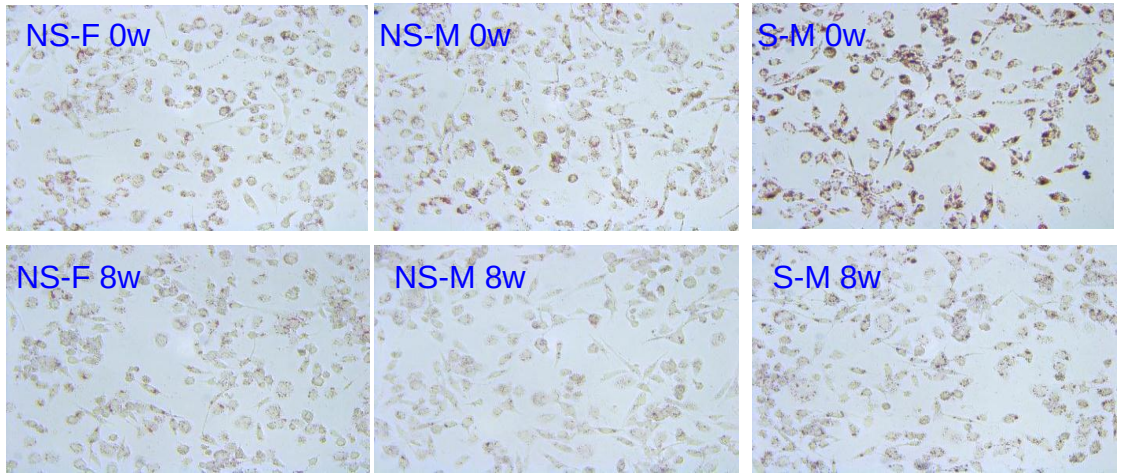
Fig. 3B





Increase of apoA-I in HDL and enhancement of anti-atherosclerotic activity by high-dose vitamin C consumption, especially male smoker group.





NS-F, non-smoker female; NS-M, non-smoker male; S-M, smoker male