TITLE

Regression of atherosclerosis with apple procyanidins by activating the ATP-binding cassette subfamily A member 1 in a rabbit model

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Abstract

Background and aims: Apple polyphenol contains abundant procyanidins, which have been associated with an anti-atherosclerosis and cholesterol-lowering effect. The aims of this study were to investigate whether apple procyanidins (APCs) features therapeutic efficacy in terms of regressing atherosclerosis and whether its efficacy is due to mechanisms other than a cholesterol-lowering effect.

Methods: After eight weeks on an atherogenic diet, rabbits were given a normal diet for another eight weeks in order to normalize the increased serum lipids level. The rabbits in the baseline group were sacrificed at this stage. The control group was subsequently fed a normal diet for eight weeks, while the APCs group was administrated 50 mg/kg/day of APCs in addition to the normal diet. Serum lipids and aortic intimal-medial thickness (IMT) were serially examined, and the resected aorta was examined histologically and through molecular biology.

Results: Aortic IMT on ultrasonography and the lipid accumulation area examined using Sudan IV staining were significantly reduced in the APCs group as compared to the control group. Serum lipid profiles were not different between the groups. Immunohistochemistry showed significantly decreased staining of an oxidative stress marker and significantly increased staining of ATP-binding cassette subfamily A member 1 (ABCA1) in the APCs group. Western blotting and RT-PCR also showed increased expression of *ABCA1* mRNA and its protein in the APCs group.

Conclusions: This study revealed that APCs administration causes a regression of atherosclerosis. APCs might hold promise as an anti-atherosclerotic agent.

Keywords

Apple polyphenol

Apple procyanidins

ATP-binding cassette subfamily A member 1

Atherosclerotic plaque regression

Anti-oxidant effect

1. Introduction

Atherosclerosis causes many cardiovascular diseases and it leads to considerable mortality and morbidity. Statin is the most representative treatment for atherosclerotic cardiovascular diseases (ACVD) and can reduce the risk of ACVD through its efficacy in lowering low-density lipoprotein cholesterol (LDL-C) [1,2]. However, statin monotherapy that includes high-dose statin sometimes fails to achieve expected LDL-C lowering in patients with ACVD [3]. Therefore, many trials have been started to develop new drugs that target not only LDL-C but also triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and lipoprotein [4,5,6].

As another approach to decreasing the risk of ACVD, polyphenols have been investigated because of their cholesterol-lowering and antioxidant properties [7]. Among polyphenols, the procyanidin group, which is widely present in vegetables and fruits including apples, in particular has potent effects that decrease the risk of ACVD [8,9]. Apples especially contain many kinds of polyphenols, with the main components being oligomeric procyanidins [10].

Many *in vitro* studies have revealed that procyanidins act in several ways that can inhibit atherosclerosis [11-17], this being confirmed by several *in vivo* studies using rodents [9,18-21]. In all *in vivo* studies, procyanidins administration was started at the same time as the atherogenic diet, which means that procyanidins were used as a preventative administration. However, therapeutic administration of procyanidins to regress already-developed atherosclerosis has not been examined. ACVDs are generally found in adults in the advanced stage of atherosclerosis;

therefore, it is meaningful to examine whether procyanidins have therapeutic efficacy for atherosclerosis. The main purpose of this study was to investigate whether apple procyanidins (APCs) can regress atherosclerosis once it has developed.

The second purpose of this study was to evaluate whether the anti-atherosclerotic effects is obtained not only by a cholesterol-lowering effect, but also through other mechanisms. Mechanisms for the anti-atherosclerotic capacity of procyanidins have been explained by *in vitro* and *in vivo* studies as the inhibition of several substances or reactions [9,11-18,21], in addition to their cholesterol-lowering effect. However, it remains unclear whether its efficacy regarding atherosclerosis is mainly attributable to mechanisms other than a cholesterol-lowering effect, since an obvious reduction in the serum LDL-C level is usually obtained by procyanidins administration [22]. Therefore, in order to evaluate mechanisms other than a cholesterol-lowering effect, a comparison should be made between a treatment group and a non-treatment group at the same serum LDL-C level. This was achieved by setting a wash-out period, providing a normal diet for eight weeks after the atherogenic diet period. As a mechanism other than a cholesterol-lowering effect, influences on ATP-binding cassette subfamily A member 1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1), which promote cholesterol efflux, oxidized low density lipoprotein (oxLDL) and lectin-like oxidized LDL receptor 1 (LOX-1), and reactive oxygen species (ROS) were focused on and examined.

2. Material and methods

2.1 Preparation of APCs

The procyanidin fraction was prepared as described below. Briefly, the apple polyphenol fraction was separated from apple (*Malus pumila* cv. Fuji) juice by preparative column with aromatic synthetic adsorbents and a Sepabeads® SP-850 (Mitsubishi Kasei Co., Ltd., Japan) column (*i.d.* 80 × 1000 mm) with the method of previous study [23]. And, apple polyphenol extracts were lyophilized, and the powder obtained was dissolved in distilled water and adjusted to pH 6.5 with 5N NaOH. The sample was applied to a Diaion HP-20ss (Mitsubishi Kasei Co., Ltd., Japan) column, and after rinsing the column with distilled water, the procyanidin fraction was eluted with 25% ethanol. Finally, the eluate was concentrated by rotary evaporation at 45°C and lyophilized as an APCs fraction. The APCs fraction powder was stored in the tightly sealed aseptic tube at 4°C before use.

2.2 Animal preparation

All experimental protocols were conducted in accordance with the Guidelines for Animal Experimentation of Hirosaki University and approved by the Animal Research Committee of Hirosaki University.

Experiments were performed using 24-week-old male Japanese white rabbits weighing 3.4 kg to 3.9 kg and purchased from Kitayama Labes Co., Ltd (Japan). They were housed under standard conditions at the Institute for Animal Experiments of the Hirosaki University School of Medicine.

2.3 Experimental Design

Nineteen rabbits were assigned randomly to three groups (Fig. 1): 1) Baseline group animals were fed a 0.3% cholesterol diet (research diets, USA) for eight weeks and then fed a normal diet for eight weeks, then sacrificed (n = 7); 2) Control group animals were fed a 0.3% cholesterol diet for eight weeks and then fed a normal diet for 16 weeks, then sacrificed (n = 7); 3). APCs group animals were fed a 0.3% cholesterol diet for eight weeks and then fed a normal diet for eight weeks, followed by a normal diet and APCs, of which 50 mg/kg were mixed with 1 ml tap water just before use and directly given by oral administration using syringes with confirmation that animals took whole doses every day for eight weeks, then sacrificed (n = 5). The APCs dosage was the same as the most effective dosage for the prevention of vasospasm of the cerebral artery after subarachnoid hemorrhage through inhibition of ox-LDL and LOX-1, as shown in our previous study using rabbits [24]. This dosage is far lower than the level with adverse effects [10].

2.4 Analysis of serum lipids and free radicals

After fasting for 12 hours, blood samples were drawn from the auricular vein and centrifuged at 3000 × g for 10 minutes to obtain sera. Serum total cholesterol (TC), LDL-C, HDL-C, and TG were measured by using a biochemistry analyzer (Hitachi 7180, Japan). The oxidative stress levels in serum were determined by the colorimetric determination of the reactive oxygen metabolites (d-ROM) test using the free-radical elective evaluator (FREE carpe diem, Diacron International, Italy). The results were expressed in arbitrary units, namely Carratelli units (U.CARR), whereby a single U corresponds to 0.08 ng/100 ml of H_2O_2.

2.5 Ultrasonography of the aorta

B-mode ultrasonography of the abdominal aorta was performed with an ultrasound machine (logiq-book XP, GE Healthcare, USA) under anesthesia by intravenous injection of medetomidine (0.5 ml/kg), midazolam (2 mg/kg) and butorphanol tartrate (0.5 mg/kg) at the beginning of the 0th week before providing a atherogenic diet and at the end of the 8th and 16th weeks in all groups, and at the end of the 24th week in the control and APCs group. Longitudinal axis images of the abdominal aorta were taken around the renal artery, and the maximal intimal-medial thickness (IMT) was measured.

2.6 Histological evaluation of atherosclerotic lesions.

At the end point for each group, the thoracic and abdominal aorta was removed just after animals were sacrificed by the administration of an overdose of anesthetics. The upper half of the thoracic aorta was used for Sudan IV staining and the lower half of the thoracic aorta and the abdominal aorta was used for HE staining, oil red staining, Masson's trichrome staining, immunohistochemistry, and molecular biological analysis.

The specimens after the Sudan IV staining were observed under a stereomicroscope (Olympus SZX7, Japan), the area of atherosclerotic plaque was measured using image analyzer Image J (National Institutes of Health, USA), and the ratio of atherosclerotic plaque area to the whole arterial area was calculated. The photographs of the specimens after HE staining, oil red staining, and Masson's trichrome staining were put under a microscope with a digital camera (DP71, Olympus, Japan).

2.7 Immunohistochemical evaluation

Immunohistochemical staining of paraffin-embedded sections was performed using Vectastain ABC Kit (Vector Laboratories, USA). After deparafinization and rehydration, heat-induced antigen retrieval was performed. Endogenous peroxidase activity was quenched using Bloxall solution (Vector Laboratories, USA). Nonspecific protein binding was blocked by normal serum, followed by the Avidin/Biotin Blocking Kit (Vector Laboratories, USA). Sections were incubated with primary antibody and biotin-conjugated anti-mouse IgG antibody or

directly with biotin-conjugated primary antibody listed in Supplemental Table 1. To visualize, sections were incubated with avidin-biotin peroxidase complexes and diaminobenzidine (Vector Laboratories, USA), then counterstained with hematoxylin. The photographs were taken under a microscope with a digital camera. In order to evaluate the detailed expression of each antigen, the observation region was divided into atherosclerotic plaque and endothelium of the non-atherosclerotic plaque. The intensity of immunohistochemical staining was examined in each region and evaluated using a semi-quantitative scoring system, by which the intensity was scored from 0 to 3 (0: none; 1: local and thin; 2: local and thick or diffuse and thin; 3: diffuse and thick for the atheroma plaque, and 0: none; 1: thin; 2: medium; 3: thick for the endothelium). Each specimen was evaluated in a blinded fashion by three independent observers.

2.8 Western blotting and real-time RT-PCR

Proteins were extracted by homogenizing in RIPA buffer containing protease inhibitors (Santa Cruz Biotechnology, Inc. USA) and centrifugation. Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies listed in Supplemental Table 2 and then with peroxidase-conjugated secondary antibodies. Bands were visualized using Amersham ECL Select Western Blotting Detection Reagents (GE Healthcare UK Ltd., UK) and ChemiDoc XRS Plus (Bio-Rad Laboratories, Inc., USA).

Total RNA was isolated using Pure Link RNA Mini Kit (Life Technologies Corporation, USA), and subjected to RT with Prime Script RT-PCR Kit (TAKARA BIO INC., Japan) using specific reverse primers. Quantitative PCR analysis was performed using iTaq Universal SYBR Green Supermix and the CFX Connect real-time PCR system (Bio-Rad Laboratories, Inc., USA). The sequences of the PCR primers were summarized in Supplemental Table 3.

2.9 Statistical Analysis

All data are expressed as means ± SEM. In the analysis of the aortic IMT, plasma lipids and d-ROM were compared with the Kruskal-Wallis test and the Wilcoxon test. In the analysis of the area of Sudan IV staining, immunohistochemical intensity score, and real time RT-PCR were compared with the Student's t test. A p-value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1 Serum lipids and d-ROMs

Serum LDL-C and TC were significantly increased after being fed the atherogenic diet in all three groups, and they significantly decreased after eight weeks of the normal diet start in all three groups (Table 1). No lipid items showed statistically significant differences among the

three groups in any period.

The serum d-ROMs increased at the 8th week after being fed the atherogenic diet and decreased at the 16th week after being fed the normal diet in all three groups without statistical significance. At the 20th and 24th weeks, the values remained low level in both the control and APCs groups (Table 1). No statistical significances were seen among the three groups in any period.

3.2 Ultrasonography of aortic IMT

Representative serial changes in the ultrasonography of the abdominal aorta in each group are shown in Figures 2A, B, C. The aortic IMT of the 8th and the 16th weeks was increased in comparison to the 0th week without statistical significance, and statistically significant differences were not seen among the three groups until the 16th week (Fig. 2D). In the APCs group, the aortic IMT at the 24th week was significantly decreased as compared to that of the 8th and 16th weeks, while in the control group the aortic IMT showed no significant differences among the 8th, 16th, and 24th weeks. At the 24th week, the aortic IMT of the APCs group was statistically significantly smaller than that of the control group (Fig. 2D).

3.3 Evaluation of aortic atherosclerosis

The representative photographs of Sudan IV staining in each group are shown in Figure 3A.

In the APCs group, the ratio of the plaque area was significantly lower than in the other two groups (Fig. 3B). Also, there was no significant difference between the baseline group and the control group. The representative findings of HE staining, oil red staining, and Masson's trichrome staining are shown in Figures 3 C, D, E. Both plaque thickness and lipid accumulation were reduced on the APCs group, while conversely collagen deposition was increased in the APCs group.

3.4 Immunohistochemistry

The microscopic findings for each staining in each group and the semi-quantitative analysis of their staining intensity are shown in Figures 4, 5, 6.

Nitrotyrosine, a specific marker for protein modification by nitric oxide-derived oxidants: at the atherosclerotic plaque, the positive-staining cells diffusely seen in the baseline group and the control group were significantly reduced in the APCs group (Fig. 4A). At the non-atherosclerotic region, the staining of the endothelial cells was significantly weaker in the APCs group as compared to the other two groups (Fig. 4B).

8-OHdG, a marker of oxidative stress to DNA: at the atherosclerotic plaque, the positive-staining cells diffusely seen in the baseline group significantly reduced in the APCs group (Fig. 4C). At the non-atherosclerotic region, the staining of the endothelial cells was significantly intense in the baseline group as compared to the other two groups (Fig. 4D).

Macrophage staining by its specific antibody, RAM11: Positively-stained cells were diffusely seen at the atheroma plaque in the baseline and control groups, and their intensity and area significantly decreased in the APCs group (Fig. 4E).

Myosin IIB, non-muscle myosin heavy chain and a marker of synthetic phenotypes of SMC: Positively stained cells were diffusely seen at the atheroma plaque in the baseline and control groups, and were significantly decreased in the APCs group (Fig. 4F).

ox-LDL and LOX-1: The positive expression of LOX-1 and ox-LDL could not be detected in all three groups (Fig. 4G, H).

ABCA1: At the atheroma plaque, the positively-stained cells were seen locally in the baseline and APCs groups, and were significantly decreased in the control group (Fig. 4I). At the non-atherosclerotic region, the staining of the endothelial cells was significantly intense in the APCs group as compared to the other two groups (Fig. 4J).

ABCG1: At the atheroma plaque, the positively stained cells were seen locally in the baseline and the APCs groups, while they could hardly be detected in the control group (Fig. 4K). At the non-atheromatous part, the staining of the endothelial cells was significantly weak in the control group compared to the other two groups (Fig. 4L).

3.5 Western blotting and RT-PCR

In western blotting, compared to the baseline group the protein expression of ABCA1 in the

control group was reduced almost completely to an undetectable level, while it was significantly preserved in the APCs group (Fig. 5A). Meanwhile, the protein expression of ABCG1 and LOX-1 were undetectable or at a slightly detectable level in all three groups (Fig. 5A).

In RT-PCR, consistent with the results of western blotting, the *ABCA1* mRNA expression of the APCs group was significantly higher than that of the other two groups (Fig. 5B). The significant differences of *ABCG1* and *LOX-1* mRNA were not seen among all three groups (Fig. 5B).

4. Discussion

In the present study, the lipid accumulation area as evaluated by Sudan IV staining, and the aortic IMT as evaluated by ultrasonography showed no significant differences between the baseline group and the control group, which suggests that atheroma once-developed is not easily regressed by providing a normal diet for a short period, such as 8 to 16 weeks. A recent experimental study using rabbit iliac artery revealed that IMT and the plaque area did not show a reduction until 16 weeks of a normal chow diet following eight weeks of an atherogenic diet, but showed significant reduction at 50 and 64 weeks [25]. Francis et al. investigated the progression and regression of aortic atherosclerotic lesions after prolonged withdrawal following four weeks of cholesterol feeding and indicated that plaque growth reached a plateau eight weeks after

cholesterol withdrawal, followed by a gradual decrease without a statistical difference until 28 weeks after cholesterol withdrawal [26]. Therefore, these results support our results, that atheroma is not easily regressed during a short period.

On the other hand, in the present study, APCs administration brought about a decrease in the lipid accumulation area and a reduction in the aortic IMT, which was accompanied by reduced infiltration of macrophage and synthetic phenotype SMCs at the atheroma plaque as evaluated by immunohistochemistry. These findings could indicate that APCs have the therapeutic efficacy to induce atherosclerotic plaque regression. This effect is considered caused not only by a cholesterol-lowering effect but also the other mechanisms, since these changes were significantly observed compared to the control group with the same serum LDL-C level as the APCs treatment group.

APCs is main component of apple polyphenols, an oligomer of epicatechin, and features strong radical scavenging activity due to its many hydroxyl groups [9,18]. The anti-oxidative stress capacity of APCs could be one of the mechanisms that regress atherosclerosis. Oxidative stress has been indicated to be an essential and initiating factor for atherosclerosis by injuring endothelial cells, oxidizing atherogenic lipoproteins and proteins, and advancing the inflammatory process [27,28]. In the present study, d-ROM, the marker of serum oxidative stress, was changed in association with the changes of serum LDL-C, which is compatible with previous studies [29,30]. Also, in the present study, d-ROM was decreased to baseline level after

the reduction of serum LDL-C in all groups, which resulted in no significant difference in the d-ROM value between the control group and the APCs group. However, immunohistochemical staining for nitrotyrosine and 8-OHdG, which are markers of local oxidative stress [31,32], were remarkably seen at the plaque and the endothelium in the baseline group and were preserved with some decreases in the control group, which suggested that local oxidative stress is maintained under normalized serum LDL-C. On the other hand, their staining was significantly decreased in the APCs group, which suggests that APCs could act as an antioxidant for the arterial wall, resulting in demonstrating an anti-atherosclerotic effect.

As with the other mechanisms, inhibition of ox-LDL and LOX-1 had been expected, since the procyanidins in APCs are indicated to be potent inhibitors of LOX-1 [33]. Ox-LDL is known to trigger up-regulation of LOX-1, and ox-LDL and LOX-1 are easily combined, resulting in increased local ROS, which play an important role in the progress of atherosclerosis [34]. However, in the present study the inhibitory effect of APCs on ox-LDL and LOX-1 could not be assessed, since their staining was not observed even in the baseline and control groups. Torzewski et al. have indicated that a significant reduction of ox-LDL at the plaque is induced by six months of a normal diet following six months of an atherogenic diet [35]. The present results suggest that ox-LDL might be decreased with down-regulation of LOX-1 in the situation of normal serum LDL-C for 8 to 16 weeks, which should be clarified in the future. Also, the inhibitory effect of APCs on ox-LDL and LOX-1 should be evaluated during a period with high

serum LDL-C.

Another effect of APCs associated with regression of atherosclerosis is its capacity to activate ABCA1 [36,37]. In foam cells, several macrophage transporters facilitate the efflux of lipids including ABCA1 and ABCG1 [38]. ABCA1 presence promotes cholesterol efflux to lipid-poor apolipoprotein A1 to form nascent HDL, and ABCG1 promotes it to mature HDL particles [38,39]. Procyanidin mainly up-regulates ABCA1 at the mRNA and protein level, independent of ABCG1 [40]. Both ABCA1 and ABCG1 exist not only in foam cells and macrophages at the plaque, but also in endothelial cells [41]. In the present study, increased immunohistochemical staining of ABCA1 was observed at the atheroma and endothelial cells in the APCs group and at the atheroma in the baseline group, which was supported by western blotting. However, the RT-PCR study showed up-regulation of *ABCA1* only in the APCs group. The expression of ABCA1 is up-regulated by the accumulation of cellular cholesterol through activation of liver X receptors (LXRs) under a high serum LDL-C level [38,42,43]. On the other hand, in the situation of a normal serum LDL-C level after a normal diet, up-regulation of *ABCA1* mRNA expression could be discontinued while its protein expression could remain with a gradual decrease, which may be a cause of the discrepancy between mRNA and protein expression of ABCA1 as seen in the baseline group. Also, both its mRNA and protein expression significantly decreased in the control group, while their expression increased again in the APCs group. APCs administration could up-regulate ABCA1 mRNA and protein under the situation of a normal

serum LDL-C level, which may be the important factor regressing the atherosclerosis in the present study. In addition, it has recently been suggested that atheroprotective effect of HDL depends on HDL functionality rather than HDL quantity [44]. APCs administration may enhance HDL quality independently of HDL quantity, since cholesterol efflux via ABCA1 is closely associated with HDL quality [45]. This hypothesis should be investigated in the next step.

The present study has suggested that APCs might have therapeutic capacity to regress already-developed atherosclerosis through mechanisms other than the cholesterol-lowering effect. The efficacy of antioxidant agents for atherosclerosis regression has not been proved in human, but APCs could be a candidate, since no observed adverse effect level for APCs has been greater than 200 mg/kg in an experimental study using rats [10].

5. Limitations

The present study has several limitations and problems to be solved in the future before APCs is clinically applicable. First, atherosclerosis obtained in the present study was not so progressive as atherosclerosis of human, since atherosclerosis was induced only by atherogenic diet in the present study. The most established atherosclerotic model in rabbits involves both balloon denudation and diet [46]. The anti-atherosclerotic efficacy of APCs should be examined using atherosclerotic model with balloon denudation and diet. Second, the present study focused only

on ABCA1and ABCG1 as a cholesterol efflux mechanism. The other mechanisms such as scavenger receptor class B type I (SR-BI) - mediated cholesterol efflux should be investigated at the next step. The detail molecular mechanism by which APCs promotes the ABCA1 expression should also be clarified, since ABCA1 is known to be regulated by upstream transcriptional factors such as LXRs and sterol regulatory element binding proteins (SREBPs). In addition, several doses of APCs should also be tested in the next study, since only one dose was evaluated in the present study. Also, an additive or synergistic effect by combined use with statin could be expected and should be investigated in order to develop a treatment for ACVD.

6. Conclusions

The present study revealed that APCs administration causes a regression of atherosclerosis in a rabbit model. Its anti-atherosclerotic efficacy is independent of a cholesterol-lowering effect. APCs might hold promise as an anti-atherosclerotic agent, although the detail mechanisms of its efficacy should be clarified in future.

Conflicts of interest

None declared.

References

1. C. Baigent, L. Blackwell, J. Emberson et al., Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials, Lancet 376(2010)1670-1681.

2. F. Barkas, M. Elisaf, H. Milionis, Statins decrease the risk of stroke in individuals with heterozygous familial hypercholesterolemia: A systematic review and meta- analysis, Atherosclerosis 243 (2015) 60－64.

3. S.M. Boekholdt, G.K. Hovingh, S. Mora, et al., Very low levels of atherogenic lipoproteins and the risk for cardiovascular events: a meta-analysis of statin trials, J. Am. Coll. Cardiol. 64(2014)485-494.

4. C.G. Santos-Gallego, J.J. Badimon, R.S. Rosenson, Beginning to understand high-density lipoproteins. Endocrinol. Metab. Clin. North Am. 43(2014)913-947.

5. M. Farnier, Future Lipid-Altering Therapeutic Options Targeting Residual Cardiovascular Risk, Curr. Cardiol. Rep. 18(2016)65.

6. R. Yahya, E. Favari, L. Calabresi, et al., Lomitapide affects HDL composition and function, Atherosclerosis 251 (2016) 15－18.

7. W Zhao, V. Haller, A. Ritsch, The polyphenol PGG enhances expression of SR-BI and

ABCA1 in J774 and THP-1 macrophages, Atherosclerosis 242 (2015) 611－617.

8. R. Corder, W. Mullen, N.Q. Khan, et al., Oenology: red wine procyanidins and vascular health, Nature 444(2006) 566.

9. J.H. Suh, A. Virsolvy, A. Goux, et al., Polyphenols prevent lipid abnormalities and arterial dysfunction in hamsters on a high-fat diet: a comparative study of red grape and white persimmon wines, Food Funct 2(2011)555-561.

10. T. Shoji, Y. Akazome, T. Kanda, et al., The toxicology and safety of apple polyphenol extract. Food Chem. Toxicol. 42 (2004) 959-67.

11. X. Terra, J. Fernández-Larrea, G. Pujadas ,et al., Inhibitory effects of grape seed procyanidins on foam cell formation in vitro, J. Agric. Food Chem. 57(2009)2588-2594.

12. H. Yang, L. Xiao, Y. Yuan,et al., Procyanidin B2 inhibits NLRP3 inflammasome activation in human vascular endothelial cells, Biochem. Pharmacol. 92(2014)599-606.

13. E. A ´ lvarez, B.K. Rodino-Janeiro, M. Jerez, et al., Procyanidins from grape pomace are suitable inhibitors of human endothelial NADPH oxidase, J. Cell. Biochem. 113(2012)1386-1396.

14. K.W. Lee, N.J. Kang, M.H. Oak,et al., Cocoa procyanidins inhibit expression and activation

of MMP-2 in vascular smooth muscle cells by direct inhibition of MEK and MT1-MMP activities, Cardiovasc. Res. 79(2008)34-41.

15. D.M. Chen, X. Cai, C.L. Kwik-Uribe, et al., Inhibitory effects of procyanidin B(2) dimer on lipid-laden macrophage formation, J. Cardiovasc. Pharmacol. 48(2006)54-70.

16. C. Quettier-Deleu, G. Voiselle, J.C. Fruchart, et al., Hawthorn extracts inhibit LDL oxidation. Pharmazie 58(2003)577-81.

17. A. Mazur, D. Bayle, C. Lab, E. Rock, Y. Rayssiguier, Inhibitory effect of procyanidin-rich extracts on LDL oxidation in vitro, Atherosclerosis 145(1999)421-422.

18. C. Auger, N. Laurent, C. Laurent, et al., Hydroxycinnamic acids do not prevent aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters, Life Sci. 74(2004)2365-2377.

19. S. Auclair, M. Silberberg, E. Gueux, et al., Apple polyphenols and fibers attenuate atherosclerosis in apolipoprotein E-deficient mice, J. Agric. Food. Chem. 56(2008)5558-5563.

20. F. Yu, B. Li, X. Li, et al., Proteomic analysis of aorta and protective effects of grape seed procyanidin B2 in db/db mice reveal a critical role of milk fat globule epidermal growth factor-8 in diabetic arterial damage, PLoS One 7(2012)e52541.

21. Z.R. Xu, J.Y. Li, X.W. Dong, et al., Apple Polyphenols Decrease Atherosclerosis and

Hepatic Steatosis in ApoE-/- Mice through the ROS/MAPK/NF- κ B Pathway, Nutrients 7(2015)7085-7105.

22. Y. Nagasako-Akazome, T. Kanda, Y. Ohtake, et al., Apple polyphenols influence cholesterol metabolism in healthy subjects with relatively high body mass index, J. Oleo Sci. 56 (2007) 417-428.

23. T. Shoji, S. Masumoto, N. Moriichi, et al., Apple (Malus pumila) procyanidins fractionated according to the degree of polymerization using normal-phase chromatography and characterized by HPLC-ESI/MS and MALDI-TOF/MS. J. Chromatogr. A. **1102** (2006) 206-213

24. N. Matsuda, H. Ohkuma, M. Naraoka, et al., Role of oxidized LDL and lectin-like oxidized LDL receptor-1 in cerebral vasospasm after subarachnoid hemorrhage, J. Neurosurg. 121 (2014) 621-630.

25. V. Khanna, M. Jain, V. Singh, et al., Cholesterol diet withdrawal leads to an initial plaque instability and subsequent regression of accelerated iliac artery atherosclerosis in rabbits, PLoS One 8(2013) e77037.

26. A.A. Francis, J.F. Deniset, J.A. Austria, et al., Effects of dietary flaxseed on atherosclerotic

plaque regression, Am. J. Physiol. Heart. Circ. Physiol. 304(2013) H1743-1751.

27. K. Husain, W. Hernandez, R.A Ansari, ,et al., Inflammation, oxidative stress and renin angiotensin system in atherosclerosis, World. J. Biol. Chem. 6(2015) 209-217.

28. I. Perrotta, S. Aquila, The Role of Oxidative Stress and Autophagy in Atherosclerosis, Oxid. Med. Cell. Longev. 2015(2015)130315.

29. N. Kabiri, S. Asgary, M. Setorki, Lipid lowering by hydroalcoholic extracts of Amaranthus caudatus L. induces regression of rabbits atherosclerotic lesions, Lipids. Health. Dis. 10(2011)89.

30. T. Sugiura, Y. Dohi, S. Yamashita, et al., Serotonin in peripheral blood reflects oxidative stress and plays a crucial role in atherosclerosis: Novel insights toward holistic antiatherothrombotic strategy, Atherosclerosis 246 (2016) 157－160.

31. H. Nishizawa, A.E. Handayaningsih, G. Iguchi, et al., Enhanced oxidative stress in GH-transgenic rat and acromegaly in humans, Growth. Horm. IGF. Res. 22(2012) 64-68.

32. A.A. Rasmiena, C.K. Barlow, N. Stefanovic, et al., Plasmalogen modulation attenuates atherosclerosis in ApoE- and ApoE/GPx 1-deficient mice, Atherosclerosis 243 (2015) 598－608.

33. T. Nishizuka, Y. Fujita, Y. Sato, et al., Procyanidins are potent inhibitors of LOX-1: a new player in the French Paradox. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci. 87(2011) 104-113.

34. M.E. Reschen, D. Lin, A. Chalisey, et al., Genetic and environmental risk factors for atherosclerosis regulate transcription of phosphatase and actin regulating gene PHACTR1, Atherosclerosis 250 (2016) 95－105.

35. M. Torzewski, P.X. Shaw, K.R. Han, et al., Reduced in vivo aortic uptake of radiolabeled oxidation-specific antibodies reflects changes in plaque composition consistent with plaque stabilization, Arterioscler Thromb Vasc Biol. 24 (2004) 2307–2312.

36. A. Chawla, W.A. Boisvert, C.H. Lee, et al., A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis, Mol. Cell 7(2001)161-171.

37. X. Terra, Juan Fernández-Larrea, G. Pujadas, et al., Inhibitory effects of grape seed procyanidins on foam cell formation in vitro, J. Agric. Food Chem. 57(2009)2588-2594.

38. K.J. Moore, F.J. Sheedy, E.A. Fisher, Macrophages in atherosclerosis: a dynamic balance, Nat. Rev. Immunol. 13(2013)709-721.

39. Y. Chi, L. Wang, Y. Liu, et al., 7-Ketocholesteryl-9-carboxynonanoate enhances ATP binding cassette transporter A1 expression mediated by PPARγ in THP-1 macrophages, Atherosclerosis 234 (2014) 467－468.

40. S.Zhao, J. Li, L. Wanga,et al.,, Pomegranate peel polyphenols inhibit lipid accumulation and enhance cholesterol efflux in raw264.7 macrophages. Food Funct. 7(2016) 3201-3210.

41. M. Westerterp, K. Tsuchiya, I.W. Tattersall, et al., Deficiency of ATP-Binding Cassette Transporters A1 and G1 in Endothelial Cells Accelerates Atherosclerosis in Mice, Arterioscler. Thromb. Vasc. Biol. 36(2016)1328-1337.

42. A.C. Calkin, P. Tontonoz, Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR, Nat Rev Mol Cell Biol.4 (2013) 213－224.

43. H. Sun, J. Shen, T. Liu, et al., Heat shock protein 65 promotes atherosclerosis through impairing the properties of high density lipoprotein, Atherosclerosis 237 (2016) 853－861.

44. CG. Santos-Gallego, HDL: Quality or quantity?, Atherosclerosis 243(2015) 121-123.

45. CG. Santos-Gallego, C. Giannarelli, jj. Badimón, Experimental models for the investigation of high-density lipoprotein-mediated cholesterol efflux, Curr. Atheroscler. Rep. 13(2011) 266-276.

46. B. Ibanez, G. Vilahur, G. Cimmino, et al., Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration:

magnetic resonance imaging study in an experimental model of atherosclerosis, J. Am. Coll. Cardiol. 51(2008) 1104-1109.

Table 1 Serial changes of serum lipids profiles and d-ROMs

	Evaluation period						
	0 week	4 week	8 week	12 week	16 week	20 week	24 week
TC (mg/dL)							
Baseline Group	23.0±1.4	461.1±122.7 [a]	540.1±112.7 [a]	105.7±33.6 [a]	30.7±7.9 [c,e]		
Control Group	32.6±4.3	455.4±88.4 [a]	792.1±215.4 [a]	188.4±82.6 [a]	41.0±17.9 [c,e]	27.3±6.5 [c,e]	22.2±1.8 [c,e]
AP Group	24.4±3.5	373.0±64.5 [a]	653.6±158.5 [a]	71.8±14.7 [a]	26.8±7.5 [c,e]	18.6±3.8 [c,e]	15.6±1.9 [c,e]
LDL-C (mg/dL)							
Baseline Group	3.9±0.7	201.1±55.2 [b]	239.1±52.5 [b]	41.3±13.8 [a]	7.3±2.9 [d,f]		
Control Group	6.4±2.4	206.1±77.9 [a]	325.6±123.1 [a]	94.0±35.5 [a]	14.3±5.4 [c,e]	8.0±3.0 [c,e]	3.4±1.3 [c,e]
AP Group	3.6±0.4	158.4±27.9 [a]	231.8±55.1 [a]	24.4±4.2 [a]	5.80±1.5 [c,e]	3.4±1.1 [c,e]	2.8±0.6 [c,e]
HDL-C (mg/dL)							
Baseline Group	11.7±0.9	26.3± 2.2 [b]	26.6±3.6 [b]	23.3±2.5 [a]	16.9±3.1 [c,e]		
Control Group	14.6±2.6	28.9± 3.2 [a]	21.0±3.5	21.6±3.7 [a]	16.4±2.0 [c]	14.5±1.7 [c]	11.0±1.4 [c]
AP Group	14.2±3.2	22.0±4.4	22.6±3.6 [a]	18.8±2.4	17.0±3.8	12.6±2.6	10.6±1.7 [e]
TG (mg/dL)							
Baseline Group	40.9±10.8	26.6±9.8	25.7±6.7	48.3±12.8	47.1±15.2		
Control Group	62.3±32.3	31.3±14.9	39.2±12.5	74.7±40.8	20.3±2.9 [e]	20.6±2.2 [e]	45.6±16.2
AP Group	49.0±17.0	20.6±6.1	36.8±16.9	53.4±36.1	17.2±3.1	23.6±2.8	35.0±8.2
d-ROMs (U.CARR)							
Baseline Group	186.3±18.5		288.4±25.4		203.4±23.2		
Control Group	205.2±33.6		255.4±24.4		239.4±72.8	171.5±11.7	162.9±9.2
AP Group	185.0±9.7		267.4±22.6		174.8±11.4	168.0±12.8	165.8±11.0

Results expressed as mean ± SEM. TC: Total Cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TG: Triglyceride, d-ROMs: colorimetric determination of the reactive oxygen metabolites. [a] $p<0.05$, [b] $p<0.01$ vs. 0 week. [c] $p<0.05$, [d] $p<0.01$ vs. 4 week. [e] $p<0.05$, [f] $p<0.01$ vs. 8 week.

Legends

Fig.1. Experimental Design

Male Japanese White rabbits of three groups were fed an atherogenic diet (0.3%cholesterol) for eight weeks to create atheroma, followed by a normal diet for eight weeks. The baseline group was sacrificed at the end of the 16th week. The control group was fed a normal diet for another eight weeks and sacrificed at the end of the 24th week. Meanwhile, the apple procyanidins (APCs) group was given a normal diet and APCs (50 mg/kg) for eight weeks and sacrificed at the end of the 24th week.

Blood sampling was performed every 4 weeks and ultrasonography of the aorta was performed every 8 weeks (arrows).

The double lines indicate time to sacrifice.

APCs: Apple procyanidins.

Fig. 2. Serial changes of aortic IMT

The representative serial ultrasonographic images of the baseline group (**A**), the control group (**B**), and the APCs group (**C**) are shown. Increased IMT (white arrows) was often seen at the 8th and 16th week in all groups.

D: Quantitative evaluation of aortic IMT. All results were expressed as mean ± SEM. *p <0.05,

**p <0.01.

IMT: intima-media thickness.

Fig. 3 Histological evaluation of aortic atherosclerosis

A: Representative findings of the thoracic aorta with Sudan IV staining.

B: Quantitative measurement of the lipid accumulation area of Sudan IV staining.

All results were expressed as mean ± SEM. *p <0.05, **p <0.01.

C: Representative findings of cross-section stained with hematoxylin/eosin.

D: Representative findings of cross-section stained with oil red.

E: Representative findings of cross-section stained with Masson's trichrome staining.

Fig. 4. Immunohistochemistry

A: Immunohistochemistry of nitrotyrosine at the atheroma plaque and its staining intensity score.

B: Immunohistochemistry of nitrotyrosine at the endothelium and its staining intensity score.

C: Immunohistochemistry of 8-OHdG at the atheroma plaque and its staining intensity score.

D: Immunohistochemistry of 8-OHdG at the endothelium and its staining intensity score.

E: Immunohistochemistry of macrophages (RAM 11) at the atheroma plaque and its staining intensity score.

F: Immunohistochemistry of myosin IIB at the atheroma plaque and its staining intensity score.

G: Immunohistochemistry of ox-LDL at the atheroma plaque and its staining intensity score.

H: Immunohistochemistry of LOX-1 at the atheroma plaque and its staining intensity score.

I: Immunohistochemistry of ABCA1 at the atheroma plaque and its staining intensity score.

J: Immunohistochemistry of ABCA1 at the endothelium and its staining intensity score.

K: Immunohistochemistry of ABCG1 at the atheroma plaque and its staining intensity score.

L: Immunohistochemistry of ABCG1 at the endothelium and its staining intensity score.

All results were expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$.

8-OHdg: 8-hydroxyguanosine; Ox-LDL: oxidized low-density lipoprotein; LOX-1: lectin-like oxidized LDL receptor-1; ABCA1: ATP-binding cassette transporter subfamily A member 1; ABCG1: ATP-binding cassette transporter subfamily G member 1.

Fig. 5. Western blotting and real-time RT-PCR

A: The results of western blotting of ABCA1, ABCG1 and LOX-1 in each group.

B: Relative expression of *ABCA1*, *ABCG1* and *LOX-1* mRNA in each group measured by real-time PCR.

All results are expressed as mean ± SEM. *P<0.05, **P<0.01.

Fig. 1 Experimental design

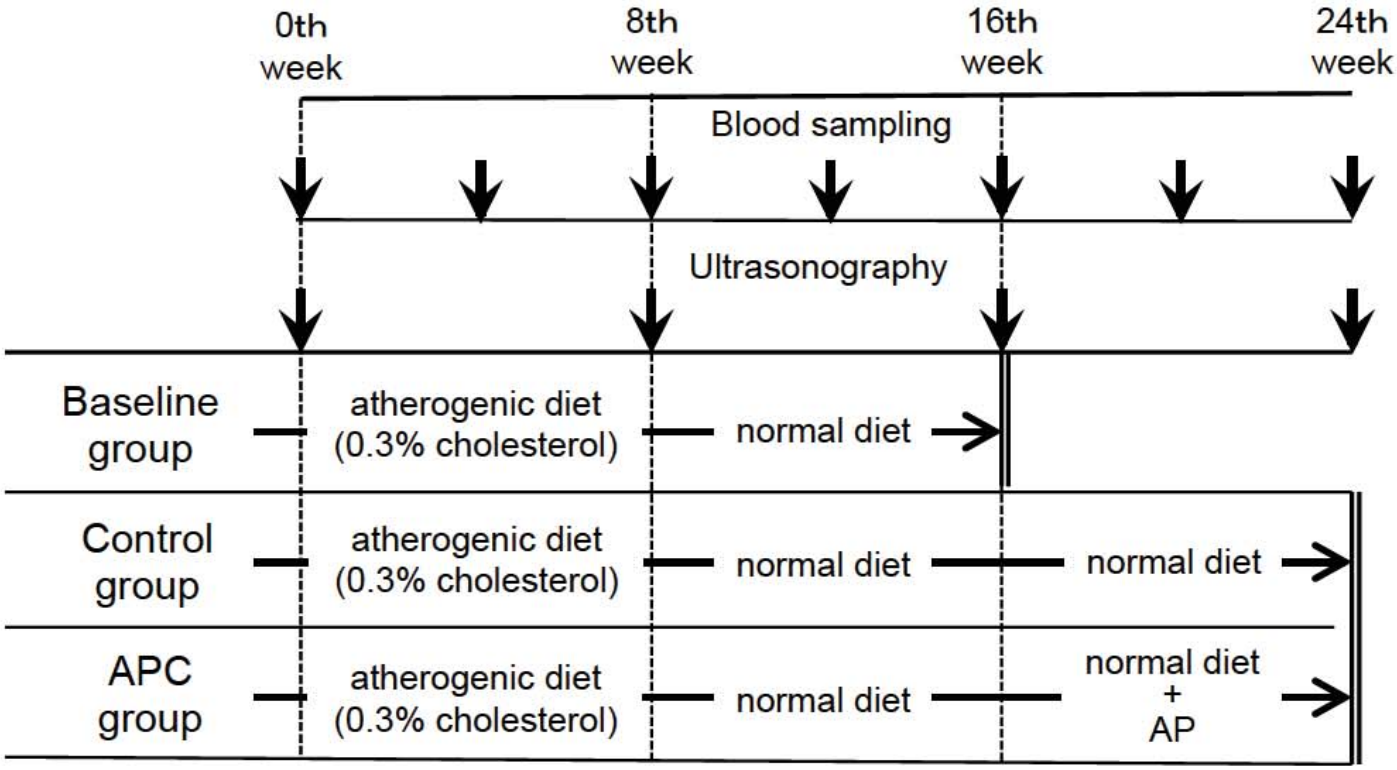
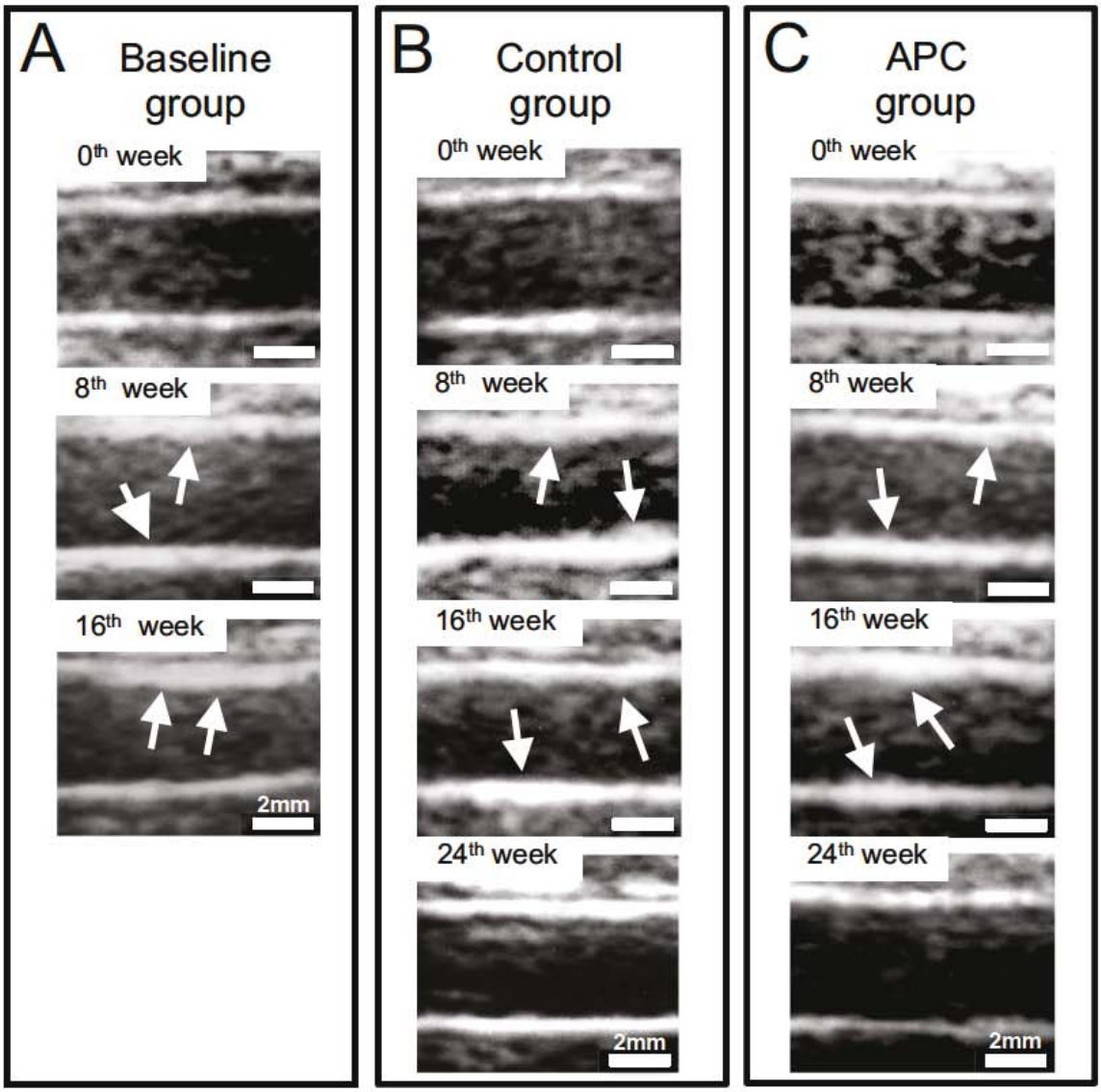


Fig. 2 Serial changes of aortic IMT



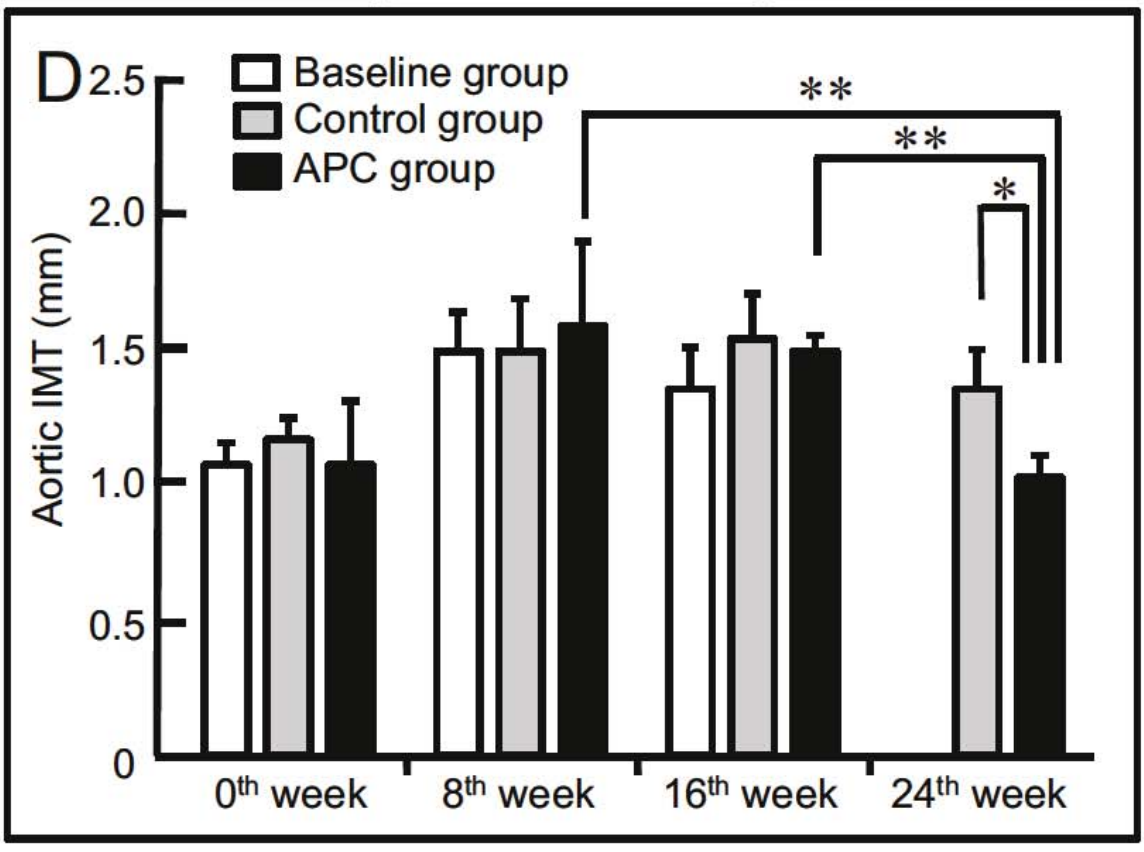


Fig. 3 Histological evaluation of aortic atherosclerosis

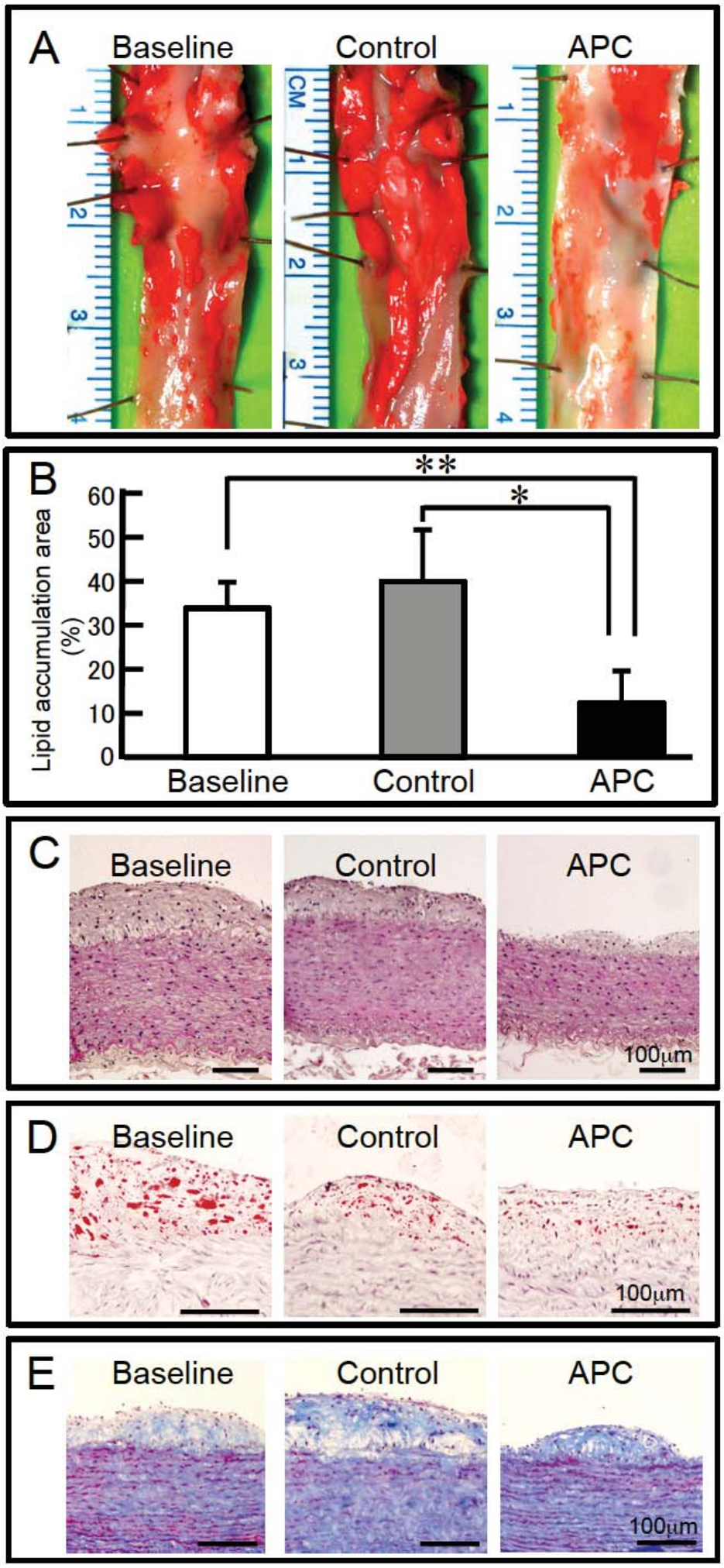


Fig. 4 Immunohistochemistry

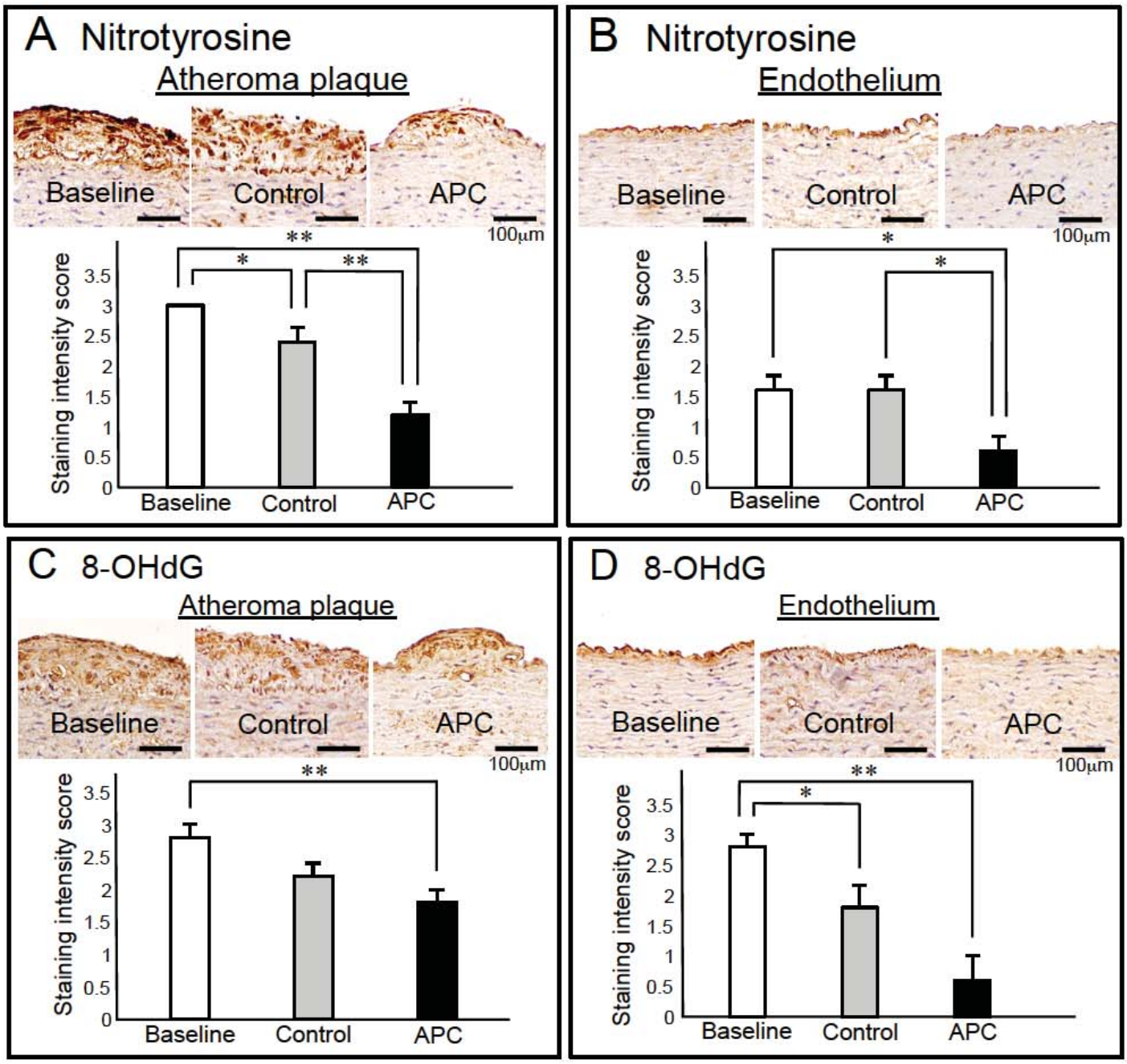


Fig. 4

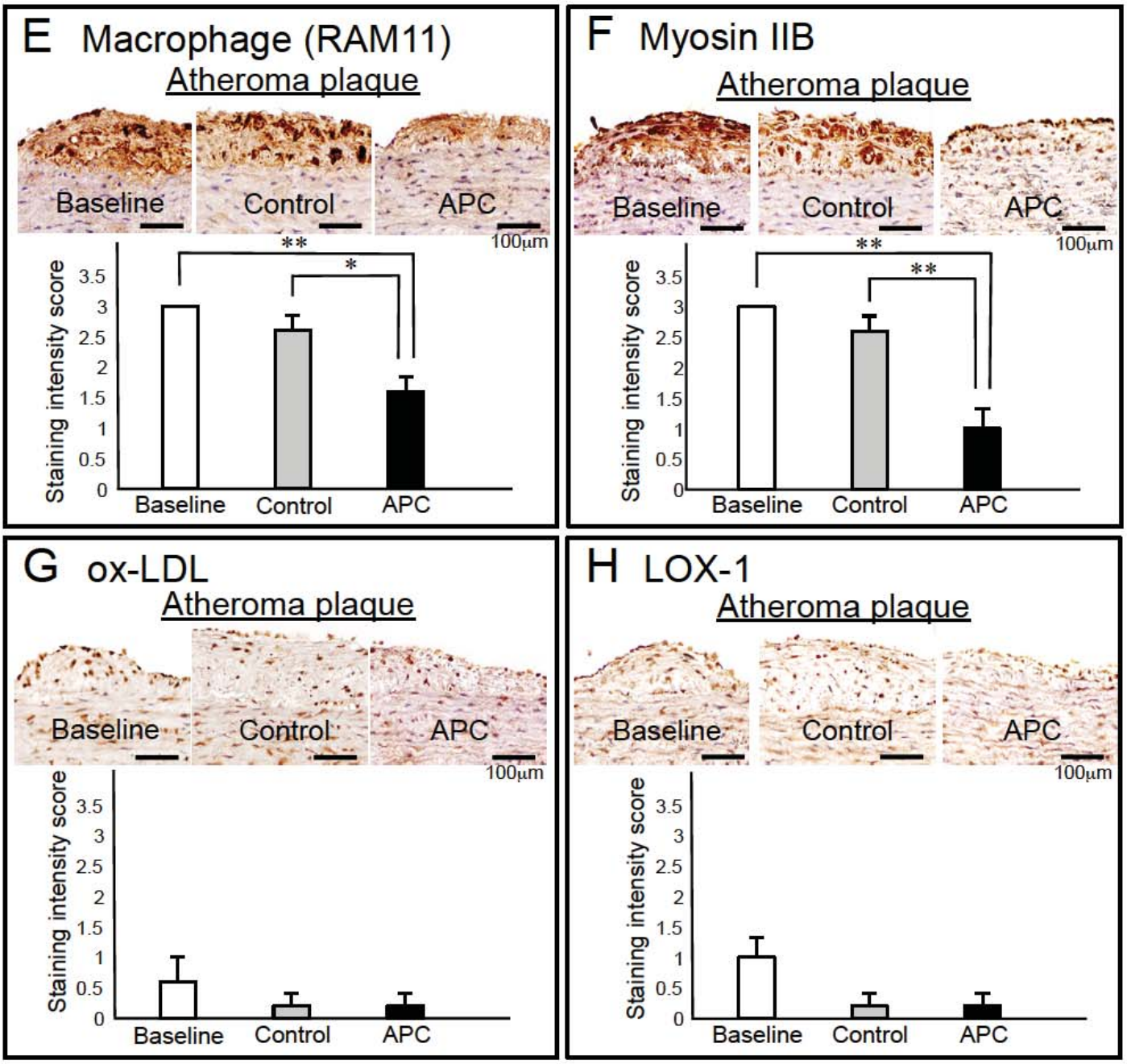

E Macrophage (RAM11)
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
3.5
3
2.5
2
1.5
1
0.5
0
**
*
Baseline
Control
APC
F Myosin IIB
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
**
**
Baseline
Control
APC
G ox-LDL
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
Baseline
Control
APC
H LOX-1
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
Baseline
Control
APC

Fig. 4

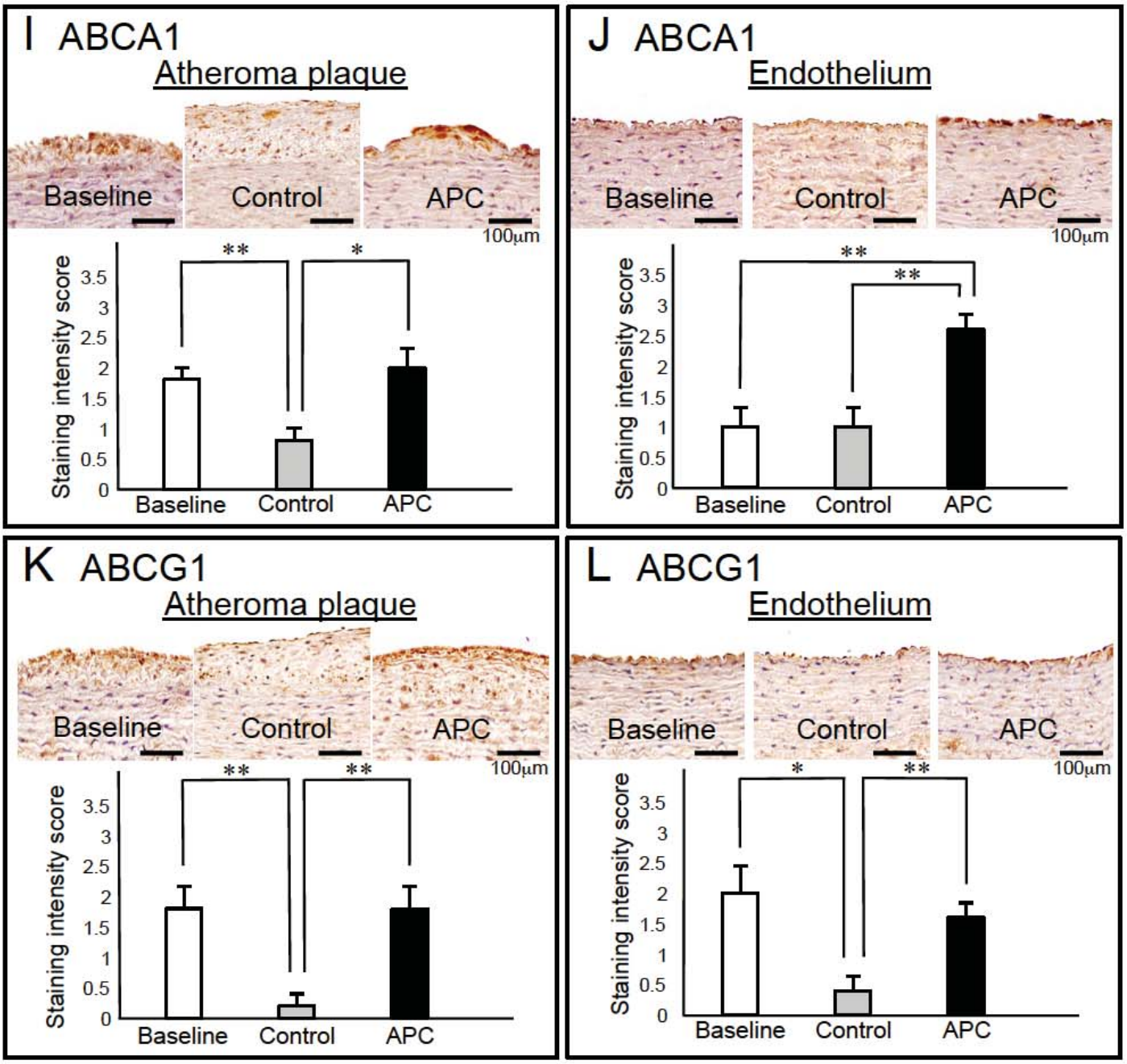

I ABCA1
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
3.5
3
2.5
2
1.5
1
0.5
0
**
*
Baseline
Control
APC
J ABCA1
Endothelium
Baseline
Control
APC
100μm
Staining intensity score
**
**
Baseline
Control
APC
K ABCG1
Atheroma plaque
Baseline
Control
APC
100μm
Staining intensity score
**
**
Baseline
Control
APC
L ABCG1
Endothelium
Baseline
Control
APC
100μm
Staining intensity score
*
**
Baseline
Control
APC

Fig. 5 Western blotting and real-time RT-PCR