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Gallic Acid Inhibits Proliferation and Migration of Smooth Muscle Cells in a Pig In-Stent Restenosis Model

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In-stent restenosis (ISR) develops primarily due to neointimal hyperplasia. Gallic acid (GA) has anti-inflammatory, antioxidant, and cardioprotective effects. This study sought to investigate the effects of GA on neointimal hyperplasia and proliferation and migration of vascular smooth muscle cells (VSMCs) in a pig ISR model. In vitro proliferation and migration experiments were confirmed, after VSMCs were treated with platelet-derived growth factor (PDGF-BB) and GA (100 μM) using a 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay and a scratch wound assay for 24 hours and 48 hours. A bare metal stent (BMS) was implanted in the pig coronary artery to induce ISR with overdilation (1.1-1.2:1), and GA (10 mg/kg/day) was administered for 4 weeks. At the 4-week follow-up, optical coherence tomography (OCT) and histopathological analyses were performed. GA decreased the proliferation of VSMCs by PDGF-BB for 24 hours (89.24±24.56% vs. 170.04±19.98%, $p < 0.001$) and 48 hours (124.87±7.35% vs. 187.64±4.83%, $p < 0.001$). GA inhibited the migration of VSMCs induced by PDGF-BB for 24 hours (26.73±2.38% vs. 65.38±9.73%, $p < 0.001$) and 48 hours (32.96±3.04% vs. 77.04±10.07%, $p < 0.001$). Using OCT, % neointimal hyperplasia was shown to have significantly decreased in the GA group compared with control vehicle group (28.25± 10.07% vs. 37.60±10.84%, $p < 0.001$). GA effectively reduced neointimal hyperplasia by inhibiting the proliferation and migration of VSMCs in a pig ISR model. GA could be a potential treatment strategy for reducing ISR after stent implantation.

Key Words: *Coronary Disease; Coronary Restenosis; Neointima; Myocytes, Smooth Muscle*

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INTRODUCTION

Percutaneous coronary intervention with stent implantation is a standard method for treating coronary artery disease in contemporary practice.[1] Although drug-eluting stents (DES) have markedly improved clinical outcomes by reducing in-stent restenosis (ISR) compared with bare-metal stents (BMS), ISR is still a challenging issue after stent implantation.[2,3] The positive effect of DES is to reduce inflammation and restenosis,[4] however, the negative effect of DES is the impairment of endothelial function, suppressed re-endothelialization, and thrombosis formation.[5,6] Although the incidence of ISR is decreased at the initial stage after DES implantation, it also increases at long-term follow-ups.[7]

The main causes of neointimal hyperplasia after coronary stent implantation are the proliferation and migration of vascular smooth muscle cells (VSMCs), as well as the production of extracellular matrix.[8] VSMCs can switch phenotypes between migration and hyperplasia in response to various pathological stimuli, leading to the development of cardiovascular diseases such as atherosclerosis and restenosis.[9,10] Platelet-derived growth factor (PDGF) is a critical factor in the proliferation and migration of VSMCs that cause neointimal hyperplasia.[11,12] Therefore, regulating the VSMCs may be a potential therapeutic method for preventing the ISR.

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Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is an abundant phenolic acid in the plant kingdom and is a compound found in green tea and wine. GA can suppress the oxidation and rancidity of fats due to its free radical scavenging and antioxidant nature, thereby promoting anti-inflammatory, antioxidant, and cardioprotective effects.[13-15] This study sought to investigate the effects of GA to reduce neointimal formation and to inhibit the proliferation and migration of VSMCs in a pig ISR model.

MATERIALS AND METHODS

1. Cell culture

Primary vascular smooth muscle cells (VSMCs) were isolated from Sprague-Dawley rat aortas (male, 8 weeks, 260 g, Orient, Korea) and Yorkshire×Landrace F1 crossbred castrated pig common carotid arteries (male, 25 kg, Chuwol grandparent farm, Korea). The endothelial layers of the arteries were scraped off using sterile forceps, and the arteries were cut into small pieces with sterile scissors. The chopped tissue was disintegrated with 0.2% collagenase type I and 0.01% elastase type II in a shaker incubator at 37℃ for 6 hours. Then, we collected the dissociated cells by centrifugation, followed by suspension in high glucose with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). All VSMCs between passages 7 and 9 were used for the experiments.

2. Cell proliferation, BrdU assay, and MTT assay in vitro

The cells were cultured in DMEM containing 10% FBS. Briefly, cells were seeded into 24-well plates at 50% confluence and maintained in 0.5% DMEM media containing 0.5% FBS for 2 days. Cells were kept in a serum-starved medium for 24 hours, and then stimulated with platelet-derived growth factor (PDGF-BB) (20 ng/mL) and GA (100 μM) for 24 and 48 hours. Finally, a 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium (MTT) assay and 5-bromo-2'-deoxyuridine (BrdU) incorporation assay were performed to determine cell proliferation of the gallic acid group. Briefly, VSMCs were treated with PDGF-BB (20 ng/mL) and GA (100 μM) for 24 and 48 hours, and then incubated with BrdU (10 mmol/L) for 80 minutes. After removing the labeling medium, the cells were washed twice with phosphate-buffered saline, fixed at room temperature for 45 minutes with 70% ethanol, and then denatured for 20 minutes with a 1 N HCl solution. After blocking with normal horse serum, the cells were incubated for 1 hour with BrdU antibody in medium. The primary and secondary antibodies were added for 1 hour, and the secondary antibody was used to detect the primary antibody. Finally, all slides were mounted with an anti-fade reagent and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), and then stained cells were counted. BrdU-positive cells (%) were expressed as the percentage of BrdU-positive cells in the total cell count.

3. Scratch wound assay

VSMCs derived from the pig's common carotid artery were used by scratch wound assay. VSMCs were seeded in 6-well plates in DMEM and serum-starved for 24 hours and then were scratched with a 200-μL pipette tip. Cells were simultaneously treated with PDGF-BB (20 ng/mL) and GA (100 μM) for 24 and 48 hours. VSMCs were visualized under a light microscope and images were captured. Image-Pro Plus software version 6.2 (Media Cybernetics, Bethesda, MD, USA) was used for quantification.

4. In-vivo study using animal model

These investigations were conducted in accordance with the guidelines of Laboratory Animals published by the US National Institutes of Health.[16] Individual timelines were applied for each pig to observe changes in physiological and functional cardiac abilities. We divided the study group [total pigs, n=10] into BMS+vehicle group (n=5) and the BMS+GA (10 mg/kg/day) group (n=5) after random BMS placement for left anterior descending artery (LAD) and left circumflex artery (LCX). After BMS was inserted into the coronary artery, the weight of the pig was measured once a week. One pig was placed in each cage to reduce stress and enable more accurate administration of medications. In the GA group, GA (10 mg/kg/day) was administered with feed for 4 weeks.

5. Preparation of pig ISR model

Yorkshire×Landrace F1 crossbred castrated male pigs (n=10, 20-25 kg) were used in the present study. Aspirin (100 mg/day) and clopidogrel (75 mg/day) were administered as a pre-treatment for 7 days. A BMS (Tiger® Stent; CG Bio, Seoul, Korea) made by the laser-cutting of a cobalt-chromium alloy tube (L605 Co-Cr alloy, 70 μm) was used in the present study. All pigs were anesthetized with zolazepam-tiletamine (2.5 mg/kg; Zoletil 50®, Virvac, Caros, France), xylazine (3 mg/kg; Rompun®, Bayer AG, Leverkusen, Germany), and azaperone (6 mg/kg; Stresnil®, Janssen-Cilag, Neuss, Germany). In addition, superficial surface electrocardiogram monitoring was performed during all experimental procedures. Lidocaine (2% solution) was injected subcutaneously into the surgical site, and the left or right carotid artery was surgically exposed. A 7 French sheath was inserted into the exposed carotid artery, and a 6 French coronary artery-guiding catheter was placed in the opening of the coronary artery. A baseline coronary angiogram was obtained using fluoroscopic guidance and a mobile fluoroscopy system (BV Pulsera, Philips Medical Systems, Andover, MA, USA). Based on angiography, a nonionic contrast agent, Omnihexol 300 (Korea United Pharm Co., Seoul, Korea), was administered, and the luminal diameters of LAD or LCX were measured by quantitative coronary analysis (QCA, CAAS, version 7.0, Pie Medical B.V., Maastricht, Netherland) using a cardiovascular angiographic analysis system. The BMS was placed by inflating the balloon (3×20 mm, nominal pressure 8 atm, 3.0 mm), and the measured luminal diameter

of the coronary artery ratio-to-stent was 1:1.1-1.2. To end the procedure, diclofenac sodium (2 mL, Depain®, Dongkwang Pharm, Korea) was injected intramuscularly pre- and post-operatively to reduce pain. After 4 weeks, the stent-grafted pigs were anesthetized (zolazepam-tiletamine 2.5 mg/kg, xylazine 3 mg/kg, and azaperone 6 mg/kg) which was followed by the final angiography. Then, the pigs were sacrificed by an intracoronary injection of 20 mL of potassium chloride.

6. Optical coherence tomography (OCT) analysis

After the follow-up angiography, a guide wire was placed in the stent-inserted coronary artery and OCT was performed using an imaging catheter (2.7 Fr C7 Dragonfly, LightLab Imaging Inc., Westford, MA, USA). OCT images were obtained by injecting iodixanol 370 (Visipaque, GE Health Care, Cork, Ireland) using a Medrad injector (Medrad Inc., Warrendale, PA, USA) by automatic pullback at a speed of 20 mm/s. OCT analysis was performed to measure the lumen area and % neointimal hyperplasia area [neointimal hyperplasia area (%)=(stent area − lumen area)/stent area×100].

7. Histopathological analysis

The coronary artery containing BMS was isolated and fixed in 4% paraformaldehyde for 48 hours. The samples were paraffin embedded and sections of 50 to 100 μm thickness were obtained at about 1 mm apart. Then, each of the slides was prepared to a 4-μm thickness using a microtome (Accu-Cut®, SRM™ 200, Sakura®, CA, USA). Each slide was stained with Hematoxylin-Eosin and Fibrin stains. Next, histopathology sections were analyzed using a calibrated microscope and microcomputer program (Visus 2000 Visual Image Analysis System, IMT Tech). The inflammation score for each stent strut was rated as follows: [0=no inflammatory cells surrounding the stent strut; 1=light, noncircumferential lymphohistiocytic infiltration surrounding the strut; 2=localized, noncircumferential, moderate-to-dense cellular aggregates surrounding the stent strut; and 3=circumferential, dense lymphohistiocytic cell infiltration of the stent strut]. The fibrin score for each individual stent strut was collected from ordinal data using a scale of 0-3 as previously reported. Immunofluorescence staining was performed using anti-CD31 antibody (1:100; ab Cambridge, MA, USA) and the secondary antibody, streptavidin Alexa Fluor® 488-conjugated anti-rabbit immunoglobulin G (1:1,000; Invitrogen, St. Louis, MO, USA). Images were obtained using a light microscopy (Eclipse 80i; Nikon, Tokyo, Japan).

8. Statistical analysis

We used the statistical package GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) for all statistical analyses. All data were expressed as the mean±standard deviation (SD). An unpaired Student's t-test was used to compare the data between the two groups for the stents. Differences in the data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons t-test, and a p value < 0.05 was considered statistically significant.

9. Ethical statement

Animal experiments were approved by the Ethics Committee of Chonnam National University Medical School and Chonnam National University Hospital (CNUHIACUC-21013).

RESULTS

1. GA decreased the proliferation of VSMCs induced by PDGF-BB in vitro

VSMC proliferation was evaluated by MTT assay. The cell proliferation was decreased in the PDGF-BB+GA group compared with the PDGF-BB group for 24 hours (89.24±24.56% vs. 170.04±19.98%, $p < 0.001$, Fig. 1A). Also, cell proliferation was decreased in the PDGF-BB+GA group compared with the PDGF-BB group for 48 hours (124.87±7.35% vs. 187.64±4.83%, $p < 0.001$, Fig. 1B). In the PDGF-BB+GA group, BrdU staining was observed in the nuclei of VSMCs (Fig. 1C), and the number of BrdU-positive cells was decreased in the PDGF-BB+GA group compared with the PDGF-BB group for 24 hours (12.87±2.46% vs. 23.87±2.62%, $p < 0.001$, Fig. 1D).

2. Scratch wound assay analysis

GA effectively inhibited the migration of VSMCs after 24- and 48-hours treatment (Fig. 2A). Migration (%) of wound closure is represented as the percentage of the vehicle scratch area in the total number of cell areas. Migration ability (% wound closure) significantly inhibited in the PDGF-BB+GA group and PDGF-BB group for 24 hours (26.73±2.38% vs. 65.38±9.73%, $p < 0.001$, Fig. 2B). Also, migration was significantly inhibited in the PDGF-BB+GA group compared with the PDGF-BB group for 48 hours (32.96±3.04% vs. 77.04±10.07%, $p < 0.001$, Fig. 2C).

3. OCT analysis

All BMSs were successfully placed in the LAD or LCX without inaccurate implantation in coronary arteries. By OCT, there were no uncovered stent struts and stent malapposition in both groups (Figs. 3A and B). A follow-up OCT image at 4 weeks confirmed the occurrence of ISR in the BMS+vehicle group (Figs. 3C and D). Neointimal hyperplasia (%) was significantly decreased in the BMS+GA group compared with the BMS+vehicle (28.25±10.07% vs. 37.60±10.84%, $p < 0.001$, Fig. 3E).

4. Histopathologic analysis

The fibrin score and the inflammation score were significantly decreased in the BMS+GA group compared with the BMS+vehicle group [0.53 (range: 0.0-1.0) vs. 2.23 (range: 1.0-2.0), $p < 0.001$] and [1.13 (range: 1.0-2.0) vs. 1.91 (range: 1.0-2.0), $p < 0.001$], respectively (Fig. 4). However, immunofluorescence staining did not significantly differ between

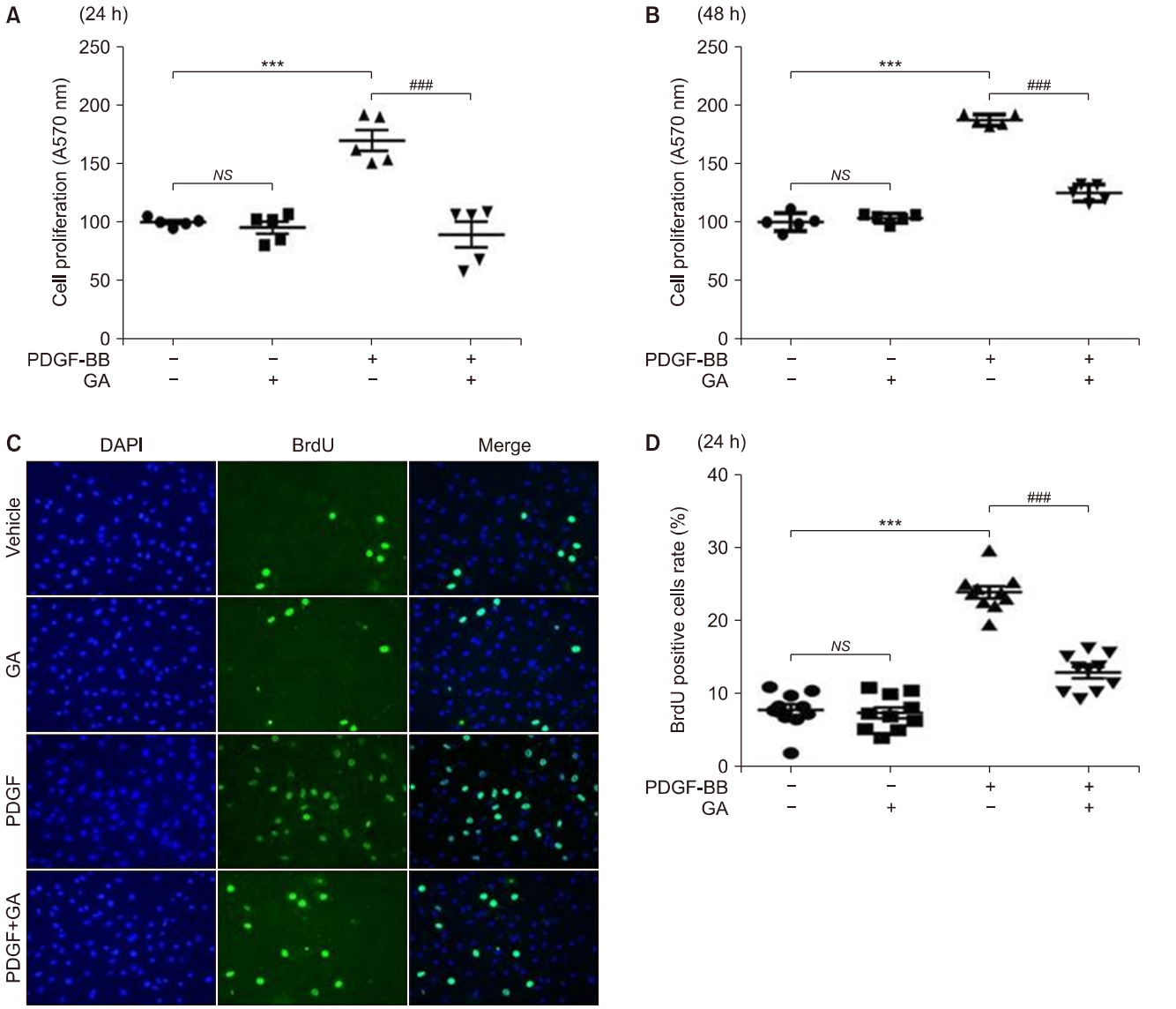

FIG. 1. Effect of gallic acid on the inhibition of platelet-derived growth factor induced vascular smooth muscle cell proliferation. The VSMCs were treated with PDGF-BB (20 ng/mL) and gallic acid (100 μM) for 24 and 48 hours, and cell proliferation was assessed by MTT assay (A, B). Immunofluorescent BrdU staining of nuclei in VMSCs treated with GA and PDGF-BB for 24 hours (C). BrdU is shown in green and DAPI is shown in blue. BrdU-positive cell rate (%) is represented as the percentage of BrdU-stained cells in the total number of cells counted (D). NS: not significant, PDGF-BB: platelet-derived growth factor-BB, GA: gallic acid.

the BMS+vehicle goup and the BMS+GA group (Fig. 5).

DISCUSSION

Inhibition of neointimal hyperplasia is a key component of preventing ISR after intracoronary stent implantation. In the present study, we simulated in vitro and in vivo models for evaluating the antiproliferative effects of GA on VSMCs and inhibition of neointimal hyperplasia. In the in vitro study, the GA significantly inhibited the proliferation and migration of VSMCs induced by PDGF-BB. In the in vivo study using a pig ISR model, GA treatment significantly inhibited the neointimal hyperplasia and inflammation at a 4-week follow-up by OCT and histopathological assay.

The main mechanism of ISR after coronary stent implantation is neointimal hyperplasia, even after DES implantation.[17,18] Incomplete endothelialization of the stent developed fibrin formations and intravascular resistance, which resulted in vascular remodeling.[19,20] The formation of neointimal hyperplasia was significantly related to the inflammatory reaction adjacent to the stent strut tissue.[21,22] Furthermore, the hyperproliferation of VSMCs is a dominant feature of neointimal hyperplasia.[23] Mechanical arterial injury during the procedure and reaction to the implanted stent cause acute or chronic inflammation of the vessel wall,[24,25] which induces multiple signaling pathways that activate VSMC migration and proliferation.[26]

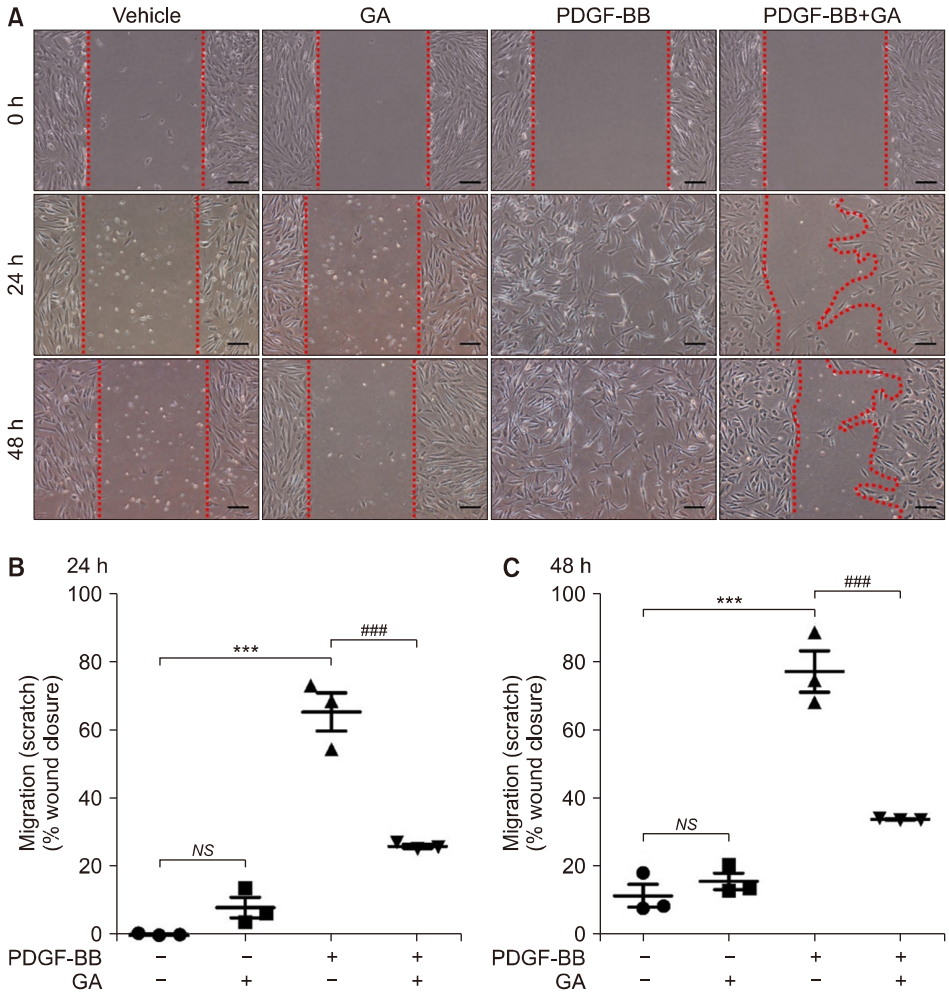

FIG. 2. Effect of gallic acid on the inhibition of vascular smooth muscle cell migration using scratch wound assay. Representative scratch wound assay images (A: magnitude×100). Migration (% wound closure) is represented as the percentage of the vehicle scratch area in the total number of cells area (B, C). NS: not significant, VSMCs: vascular smooth muscle cells, GA: gallic acid.

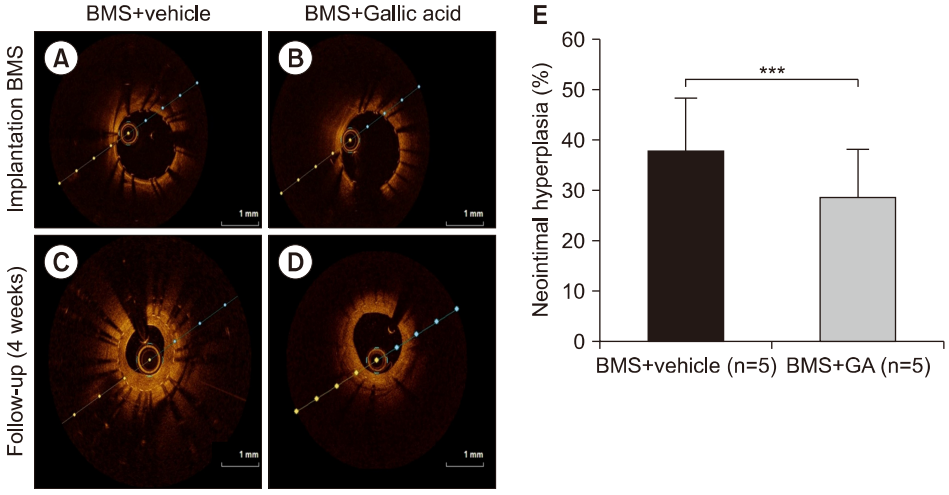

FIG. 3. Representative optical coherence tomography images. Post-BMS implantation OCT images (A, B) and at the 4 weeks follow-up OCT images (C, D). percentage neointimal hyperplasia (E). BMS: bare metal stent, OCT: optical coherence tomography.

The PDGF receptor is an important factor in the proliferation and migration pathways of VSMCs and is a major factor in reducing restenosis by inhibiting PDGF signaling.[27] Recent studies have continuously highlighted the importance of the PDGF receptor signaling pathway in the pathophysiology of atherosclerosis and restenosis.[28,29]

GA is a 3,4,5-trihydroxybenzoic acid found in tea leaves and some fruits and has been known to suppress the oxidation and rancidity of fats due to its free radical scavenging and antioxidant nature, thereby promoting anti-inflammatory, antioxidant, and cardioprotective effects.[30,31] Recently, there has been increasing evidence that GA is bene-

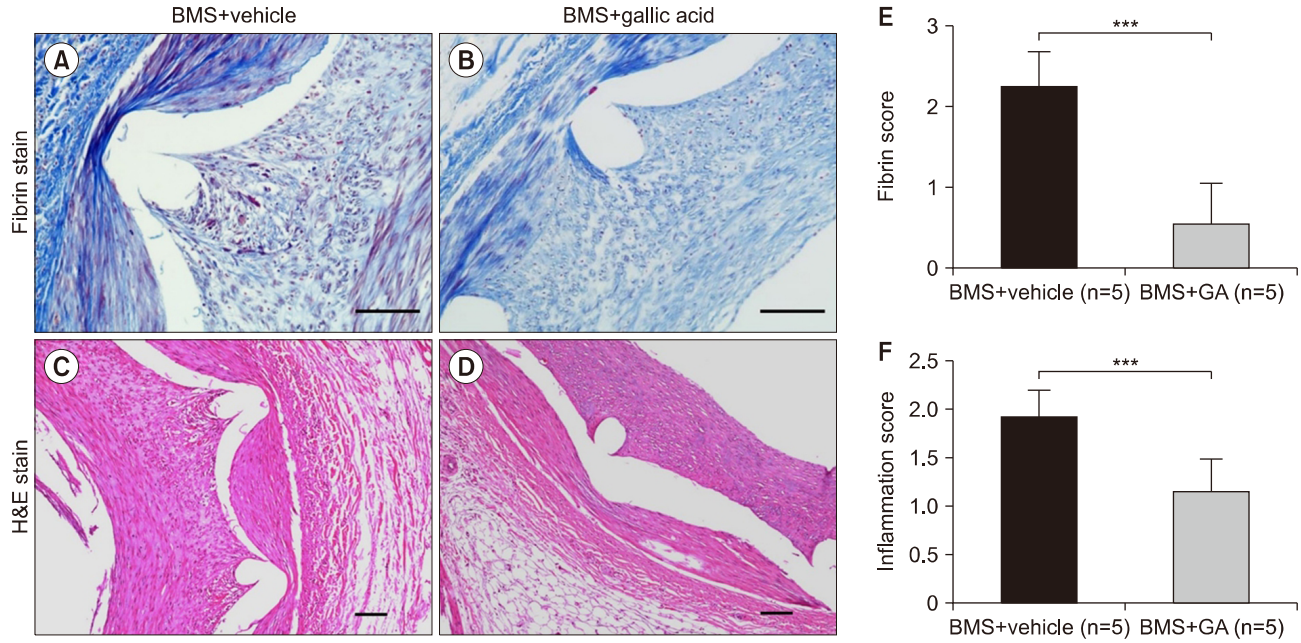

FIG. 4. Histopathology analysis. Representative histopathology images of fibrin stain (A, B: magnitude×200) and hematoxylin and eosin stain (C, D: magnitude×100) at 4 weeks after stenting. Fibrin score (E), inflammation score (F).

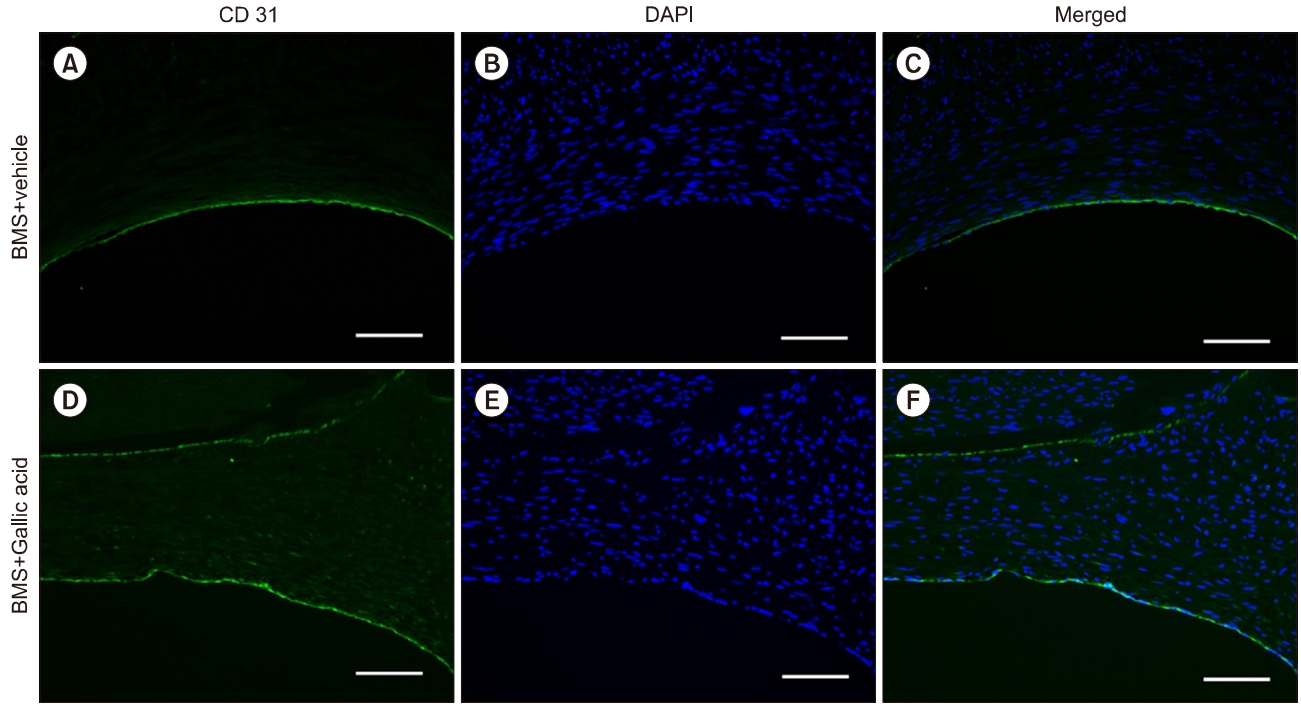

FIG. 5. Immunofluorescence staining of endothelial cell formation at 4 weeks follow-up. Immunofluorescence staining images of CD31 (A, D), DAPI stain (B, E), and merged images (C, F) at 4 weeks after stenting (magnitude×200). BMS: bare metal stent.

ficial for cardiac hypertrophy and fibrosis,[32] vascular calcification,[33] hypertension,[34] and oxidative stress.[35] In the current study, GA significantly inhibited the proliferation of VSMCs induced by PDGF after 24- and 48-hour treatments as assessed by the MTT and BrdU assays. In addition, GA inhibited the migration of VSMCs in a scratch wound assay after 48-hour treatment. In the in vivo study using a pig ISR model, we have confirmed the antiproliferative effects of GA on neointimal hyperplasia. At the 4-week follow-up after BMS implantation, GA significantly reduced neointimal hyperplasia and inflammation. These results indicate that GA may be a potential treat-

ment option for reducing neointimal hyperplasia.

This study has several limitations to be addressed. First, the doses of GA used in animal experiments, 10 mg/kg/day, were selected at the investigators' discretion, and there was a lack of pharmacokinetic data. High-dose administration of GA has demonstrated its safety and toxicity range from 100 to 300 mg/kg/day.[36] Since we focused on the safety and toxicity of GA rather than efficacy in the vivo study, the dose might not be problematic. Second, the VSMCs used in the in vitro experiments were isolated from the aortic arch of rats and the common carotid artery of pigs. Therefore, it should be noted that the results in this study likely can not be generally applied to human VSMC. Third, the dose of GA for humans could not be determined based on the current study. Fourth, although antioxidant and anti-inflammatory parameters could provide evidence of mechanistic links for the suppression of ISR, this information was not available in the current analysis. Further studies are required to evaluate the clinical relevance of GA in reducing ISR.

In conclusion, GA effectively inhibits the proliferation and migration of VSMCs and finally reduces neointimal hyperplasia and inflammation in a pig ISR model. GA might be a potential treatment option for reducing ISR after intracoronary stent implantation.

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CONFLICT OF INTEREST STATEMENT

None declared.

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