Controlled Release of Ascorbic Acid from Gelatin Hydrogel Attenuates Abdominal Aortic Aneurysm Formation in Rat Experimental Abdominal Aortic Aneurysm Model

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(Degree)
博士（医学）

(Date of Degree)
2013-10-09

(Date of Publication)
2014-10-01

(Resource Type)
doctoral thesis

(Report Number)
乙第3231号

(URL)
https://hdl.handle.net/20.500.14094/D2003231

Controlled Release of Ascorbic Acid from Gelatin Hydrogel Attenuates Abdominal Aortic Aneurysm Formation in Rat Experimental Abdominal Aortic Aneurysm Model

ゼラチンハイドロゲルからのアスコルビン酸の徐放は

ラット腹部大動脈瘤実験モデルにおける腹部大動脈瘤形成を減弱させる

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Key words: abdominal aortic aneurysm, ascorbic acid, gelatin hydrogel, controlled release, antioxidant effect, anti-inflammatory effect

ABSTRACT

Objective: Abdominal aortic aneurysms (AAAs) are associated with oxidative stress and the inflammatory response. We investigated the hypothesis that the known antioxidant ascorbic acid, which can also promote elastin and collagen production by smooth muscle cells, would prevent AAA formation in a rat model.

Methods: An intraluminal elastase and extraluminal calcium chloride-induced rat AAA model was used, and the animals were divided into 3 groups: control (Group C, N = 18), the aorta wrapped with saline impregnated gelatin hydrogel sheet (Group G, N = 18), and the aorta wrapped with gelatin hydrogel sheet incorporating ascorbic acid (Group A, N = 18). Wrapping of the sheet was completed at the end of treatment for AAA creation. Aortic dilatation ratio was measured and aortic tissues were further examined for oxidative stress and oxidative DNA damage using biochemical and histological techniques.

Results: Aortic dilatation at both 4 and 8 weeks was inhibited in Group A (dilatation ratio (%) at 4 weeks: 186.2 ± 21.8 in Group C, 152.3 ± 10.2 in Group G, 126.8 ± 11.6 in Group A, $P < .0001$; dilatation ratio (%) at 8 weeks: 219.3 ± 37.5 in Group C, 194.0 ± 11.6 in Group G, 145.7 ± 8.3 in Group A, $P = .0002$). Elastin and collagen content were significantly preserved in Group A (elastin: $P = .0015$; collagen: $P < .0001$). The messenger RNA (mRNA) expressions of matrix metalloproteinase (MMP)-9, monocyte chemotactic protein-1, interleukin-1β and tissue necrosis factor-α ($P = .0024$, $P < .0001$, $P < .0001$, $P < .0001$, respectively) was down-regulated in Group A ($P = .0024$), while tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 were both up-regulated in Group A (TIMP-1: $P = .0014$; TIMP-2: $P < .0001$). Gelatin zymography showed activities of pro MMP-2, MMP-2 and MMP-9 were significantly suppressed in group C ($p< .0001$ for each). Reactive oxygen species (ROS) expression and 8-hydroxydeoxyguanosine (8-OHdG) and cluster of differentiation (CD) 68 staining were

significantly suppressed in Group A (ROS expression: $P < .0001$; 8-OHdG positive cells: $P < .0001$; CD68 positive cells: $P < .0001$)

Conclusions: Controlled release of ascorbic acid using gelatin hydrogel sheet attenuated AAA formation through antioxidant and anti-inflammatory effect, regulation of MMP-2, TIMP-1 and TIMP-2, and preserving elastin and collagen in this animal model.

Clinical relevance

Current options for treating AAAs are limited to open or endovascular repair. A simple method other than replacement would be highly beneficial to patients. Wrapping the dilated aorta has been reported to be effective in preventing future dilatation and rupture. Gelatin hydrogel is not only suitable for wrapping vessels, but also is biocompatible and does not cause inflammatory changes. Furthermore, it can deliver drugs directly to the target organ over period of time with superior effect than systemic application. Application of ascorbic acid impregnated gelatin hydrogel sheet may enable to attenuate the future dilatation of AAAs in human.

INTRODUCTION

The pathogenesis of abdominal aortic aneurysms (AAA) has been examined in the past decades. Inflammation and tissue degeneration have been proven to play key roles in this process.[1] Moreover, the enhanced oxidative stress that occurs during the inflammatory response has been demonstrated to contribute to the formation of AAAs. [2, 3]

Ascorbic acid has powerful antioxidant properties and can protect tissue from oxidative damages.[4] This compound provides a vascular defense against oxidative stress by scavenging free radicals and by increasing endothelial nitric oxide synthase activity.[5] In addition, ascorbic acid has been reported to enhance elastin and collagen production from aortic smooth muscle cells, and these two proteins have been demonstrated to be crucial components of the vascular structure in previous *in vitro* studies. [6, 7, 8]

Gelatin hydrogel sheet has been demonstrated to provide stable release of several substances[9, 10, 11] and to have excellent vessel fitting characteristic, with the effect of circumferential mechanical support, improving structural and physiological properties.[12] This material and methodology can prolong the effect of the medication in a target tissue selectively and continuously. Furthermore, it has a great potential to provide superior therapeutic efficacy for topical pharmacological applications on aneurysmal vessels compared with systemic application.[13]

In the current study, we hypothesized that wrapping gelatin hydrogel sheet to provide the sustained release of ascorbic acid could attenuate aneurysmal development by both protecting against oxidative stress and by promoting elastin and collagen production.

METHODS

In vivo controlled release of ascorbic acid from gelatin hydrogel sheet

The gelatin used in the present study was isolated using an alkaline process from bovine bone with an isoelectric point of 4.9 and molecular mass of 99 000 kDa (Nitta Gelatin Co, Osaka, Japan). The gelatin sheets were prepared by chemical cross-linking of a 5% bovine bone gelatin solution with glutaraldehyde. Gelatin sheets of 20 mm^2/0.3 mm were prepared and immersed in 50 mmol/L glycine aqueous solution at 37 °C for 1 hour to block the residual aldehyde groups of glutaraldehyde. The sheets were then freeze-dried and sterilized by ethylene oxide gas. Ascorbic acid was incorporated into the gelatin hydrogel sheets by immersion in a 200 mg/ml ascorbic acid solution for 30 minutes. The release test was performed in a 1mL phosphate buffered saline solution containing 0.01 weight% TWEEN® 80 (Sigma-Aldrich, Tokyo, Japan) by soaking a 5 mm by 5 mm ascorbic acid-impregnated gelatin hydrogel sheet to assess the concentration of ascorbic acid at 12, 24, 48, 72, 96 and every 48 subsequent hours, up to 336 hours. The sheet was removed at each endpoint, and the solution was combined with 1 mL of 10% metaphosphoric acid and centrifuged. The supernatant was used for the assay using a Vitamin C Assay Kit (Cosmo Bio Co, LTD, Tokyo, Japan), following the instructions for the use. An oxidizing agent was added to each sample, which was incubated for 5 minutes at room temperature. Next, 5% metaphosphoric acid/2% stannum chloride was added to the sample. After the addition of 2, 4- dinitrophenylhydrazine to the sample and 3 hours of incubation at 37 °C, 85% sulfate was added. Lastly, the samples were transferred to 96–well plate, and their absorbances were measured at 530 nm. To determine the amount of ascorbic acid in each sample, the absorbance value of each sample was compared with a set of standard ascorbic acid samples that were prepared in parallel.

In vivo controlled release of ascorbic acid from gelatin hydrogel sheet

A 5 mm by 10 mm ascorbic acid-impregnated gelatin hydrogel sheet was implanted subcutaneously in 4 separate parts of the body, left and right side of the back and chest and

abdomen, in 3 rats to assess the concentration of ascorbic acid in tissue. At 10, 24, 108 and 216 hours, the sheet was removed and the subcutaneous tissue was taken and was weighed. Then the sample was homogenized with 5.4% metaphosphoric acid and centrifuged. The supernatant was used for the assay using a Vitamin C Assay Kit (Cosmo Bio Co, LTD, Tokyo, Japan).

Animal Model

In the present study, 8-week-old male Sprague-Dawley rats (SLC Inc., Shizuoka, Japan) (N = 54) were used and handled according to the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine (permission number P080120) and the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/5140.html).

The experimental AAA saccular model was described previously.[14] Briefly, a 1-cm segment of the infrarenal abdominal aorta was exposed via a midline laparotomy. Subsequently, the aorta was filled with 30 μl (30 U) of porcine pancreatic elastase (135 U/mg; Elastin Products Company, Owensville, USA) and 0.5 M $CaCl_2$ (Sigma-Aldrich, Tokyo, Japan) soaked gauze was wrapped around the region simultaneously for 20 minutes. The abdominal incision was closed in layers, and rats were allowed to recover.

Ascorbic acid impregnate hydrogel sheet administration

Ascorbic acid was incorporated into the gelatin hydrogel sheets by immersion in a 200 mg/ml ascorbic acid solution for 30 minutes prior to implantation. Following elastase infusion and calcium chloride exposure of the abdominal aorta, the rats were further treated according to each group. For Group C, (control group), the abdomen was closed in layers without any additional procedure. For Group G, the treated aorta was wrapped circumferentially with a 5 mm by 10 mm plain gelatin hydrogel sheet that was submerged in natural saline for 30 minutes. For Group A, the treated aorta was enveloped with a 5 mm by 10 mm ascorbic acid incorporated gelatin hydrogel sheet.

The 1-week samples (N = 6 in each group) were used for the molecular analyses, immuohistochemical analyses and *in situ* imaging. The 4-week samples (N = 6 in each group) were used for the morphometric, histologic and quantitative analyses. The 8-week samples (N = 6 in each group) were used for the morphometric analyses.

Macroscopic assessments

The animals were anesthetized, and the abdominal aorta was exposed and photographed using a digital camera (Leica IC D, Leica Microsystems, Wetzlar, Germany). The maximal dimension of the transverse minor axis of the abdominal aorta and the aortic diameter at the infrarenal proximal neck site were measured under physiologic conditions using an optical micrometer prior to isolation. The dilation ratio was calculated according to the following formula: dilation ratio (%) = [maximal aneurysm diameter / native aortic diameter] X 100. AAA was defined as when the dilation ratio exceeded 150%.

Specimen preparation

The treated 1-cm aortic segments in each group were isolated at each study endpoint. The 1-week aortic segment was cut into two equal portions transversely. One segment was used for messenger RNA (mRNA) analysis, and the remaining portion was further bisected for the immunohistochemical analysis. The first portion was fixed in 10% formalin, dehydrated in a graded ethanol bath, cleaned in xylene, and paraffin-embedded. The remainder was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co, Tokyo, Japan) in liquid nitrogen and stored at -80 °C until required. The 4-week aortic segments were bisected for histological assessment and quantification of elastin and collagen. The 4-week aortic segments for the histological analysis were fixed in 10% formalin, dehydrated in a graded ethanol bath, cleaned in xylene, and paraffin-embedded.

Histology

Paraffin-embedded 5 μm-thick sections were stained by hematoxylin and eosin (H&E) for general appearance, with elastic Van Gieson (EVG) for elastin, and with picrosirius red (PSR) for collagen. The images for the sections were captured with a microscopic system Biozero (KEYENCE Co, Osaka, Japan) and were assessed using BZ-H1M (KEYENCE) and Image J 1.41 software (National Institute of Health, Bethesda, MD). The areas of collagen in a cross-sectional aortic wall were semiquantified using Image J plug-in Color Counter with a color threshold mask. The collagen content was calculated by dividing the collagen-positive area by the cross-sectional aortic wall area in the PSR-stained sections. The method that was used for this analysis was described by Perrée et al.[15] The collagen content was expressed as a percentage of the total area.

Quantification of elastin

The rat aortic elastin content was quantified using the Fastin elastin assay kit (Biocolor; www.biocolor.co.uk), following the manufacturer's instructions. Briefly, the rat aorta samples were homogenized in 0.25 M oxalic acid. After centrifuging, the supernatants were treated with an elastin-precipitating reagent and centrifuged. After discarding the supernatants, the elastin pellet was treated with a dye reagent that binds to elastin. Lastly, the bound dye-elastin complex was resuspended, and the dye was disassociated from the elastin. The absorbance of each sample was measured at 513 nm and compared with a set of standard elastin samples. The elastin content of each rat aorta sample was expressed as a percentage of the dry weight of the vessel.

Immunohistochemistry

The immunohistochemical staining was performed on paraffin-embedded sections of 1-week samples of the aorta using monoclonal antibody that is specific for 8-hydroxydeoxyguanosine (8-OHdG) (Japan Institute for the Control of Aging, Shizuoka,

Japan), which marks oxidative DNA damage,[16] for cluster of differentiation (CD) 68 for macrophages and for monocyte chemotactic protein (MCP)-1 (Santa Cruz Biotechnology, Inc., Dallas, TX, US). Briefly, deparaffinized sections were incubated overnight at 4 °C with a primary antibody against 8-OHdG (5.0 μg/ml), CD68 (1.0 μg/ml) and MCP-1 (4.0 μg/ml). Following three washes, the sections were incubated at room temperature for 30 minutes in a solution with an anti-mouse IgG secondary antibody (K4001; Dako). The microscopic digital images of five random fields on each section were scanned in a frame that was composed of 500 μm by 380 μm rectangles, and the number of 8-OHdG-positive cells and CD68-positive cells in aortic wall was counted using Dynamic cell count BZ-H1C software (KEYENCE).

In situ superoxide Imaging

The 1-week samples of aortic wall embedded in Tissue-Tek® O.C.T. Compound were cut into 10 μm sections. Dihydroethidium (DHE) (Invitrogen, Tokyo, Japan) was used to evaluate tissue superoxide levels *in situ*, as previously described.[17] The sections were incubated with DHE in phosphate-buffered saline in a dark, humidified chamber for 30 minutes at 37°C. DHE fluorescence was detected through a 580 nm filter, and DHE fluorescence images of five randomly selected fields on each section were scanned with Biozero software in a frame that was composed of 500 μm by 380 μm rectangles. DHE fluorescence of the aortic sections was quantified using Dynamic cell count BZ-H1C software. The mean fluorescence was semiquantified and expressed relative to the values that were obtained for native rat tissue.

Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from graft samples using an RNeasy fibrous tissue mini-kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The RNA was transcribed and amplified to complimentary DNA (cDNA) using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time

polymerase chain reaction (PCR) analyses for mRNA levels of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1, TIMP-2, interleukin (IL)-1β and tumor necrosis factor (TNF)-α were performed using an ABI Prism 7500 sequence detection system (Applied Biosystems) with TaqMan universal PCR master mix (Applied Biosystems) and TaqMan real-time PCR primers (Applied Biosystems). The expression levels of each mRNAs were divided by the mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Gelatin zymography

Proteins from aortic specimens were extracted using a buffer containing 50mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.2% sodium dodecyl sulfate, and 1mM ethylenediaminetetraacetic acid, supplemented with protease inhibitors (20 μg/mL aprotinin,10 μg/mL leupeptin, and 1mM phenylmethylsulfonyl fluoride). To determine gelatinolytic activities of MMP-2 and MMP-9 in the treated aorta, gelatin-zymography kit (Primary Cell Co, Hokkaido, Japan) was used according to the manufacturer's instructions. The protein concentration was standardized with a microbicinchoninic acid protein assay kit (Pierce, Rockford, Ill), and then protein (20 μg) was applied in each lane for the electrophoresis. Densitometric analysis of the lytic bands was performed by Image J software.

Statistical analysis.

The database management and statistical analysis were performed using JMP software (SAS Institute Inc., Cary, NC, USA). All of the values are expressed as the means ± standard deviation of the mean of the obtained values for each rat in a given group. The comparisons between multiple groups were performed using one-way analysis of variance, followed by the Tukey-Kramer honestly significant difference test. A value of $P < .05$ was considered to be statistically significant.

RESULTS

Release test

In vitro release test demonstrated that ascorbic acid was rapidly released from gelatin hydrogel sheet during the first 48 hours and was gradually released over a period of several weeks (Fig. 1A). The concentration of ascorbic acid reached 30 μg/ml and the sheet did not dissolve in phosphate-buffered saline at the completion of the 14-day release test. On the contrast, *in vivo* release test showed the ascorbic acid concentration of tissue was maintained around 100μg/ml for days (Fig. 1B). The actual sheet wrapped around the abdominal aorta in rat retroperitoneum was clearly present at 1 week, and was barely detected at 4 weeks, then completely dissolved at 8 weeks.

Macroscopic assessments

Aneurysm formation was observed at 4 and 8 weeks. The macroscopic findings are given in Figure 2. According to the AAA definition in the present study, AAAs formed in 100% of the rats in Group C at both 4 and 8 weeks. Among rats in Group G, 83.3% exhibited AAA formation at 4 weeks, and 100% exhibited AAAs at 8 weeks. None of the rats in Group A had developed AAAs at 4 weeks, and only 16.7% formed AAAs at 8 weeks. Significant differences were observed in the AAA formation rates at 4 and 8 weeks in Group A compared with Groups C (at 4 weeks, $P = .0005$; at 8 weeks, $P < .0001$) and G (at 4 weeks, $P = .0034$; at 8 weeks, $P = .0118$). The dilation ratios of the AAAs at 4 and 8 weeks were also significantly lower in Group A (126.8 ± 11.6% at 4 weeks, 145.7 ± 8.3% at 8 weeks) than those that were observed in Groups C (186 ± 21.8% at 4 weeks, $P < .001$; 219.3 ± 37.5% at 8 weeks, $P < .0001$) and G (152.3 ± 10.2% at 4 weeks, $P = .0034$; 194.0 ± 11.6% at 8 weeks, $P = .0025$).

Elastin and collagen

EVG staining revealed various degrees of degeneration and disruption of the elastic lamellae in all the Groups C, G and A (Fig. 3). But the elastin content in Group A was significantly preserved compared with Groups C and G (elastin (μg/tissue weight mg): 31.0 ± 1.6 in Group C, 37.3 ± 6.9 in Group G, 47.4 ± 11.8 in Group A, Fig. 4A).

PSR staining demonstrated preserved wall thickness, with high collagen contents, in Group A compared with the other two groups (Fig. 3). The semi-quantification of collagen content revealed that Group A contained significantly higher collagen levels compared with Groups C and G (collagen positive area (%): 4.7 ± 0.4 in Group C, 3.9 ± 0.4 in Group G, 9.2 ± 0.4 in Group A, Fig. 4B).

Antioxidant Effect of ascorbic acid

The DHE staining revealed that relative ROS expression in the aortic wall was significantly suppressed in Group A, especially in the outer layer of the aorta (Fig. 5A), compared with Groups C and G (4.6 ± 0.7 in Group C, 4.2 ± 0.4 in Group G, 1.8 ± 0.2 in Group A, Fig. 5B). Similarly, the number of 8-OHdG-positive cells in the aortic walls was significantly decreased in Group A compared with Groups C and G (131.3 ± 16.1 cells in Group C, 121.8 ± 16.1 cells in Group G, 75.3 ± 24.2 cells in Group A, Fig. 5A and 5C).

Inflammatory response

Inflammation is believed to contribute to the etiology of MMP-regulated and TIMP-regulated AAA formation. The mRNA expression levels of MMP-2 were similar in all 3 of the groups (Fig. 6A). However, the mRNA expressions of MMP-9 were significantly down-regulated in Group A compared with both Groups C and G (2.36 ± 0.30 in group C, 2.30 ± 1.52 in group G, 0.40 ± 0.17 in group A). In contrast, the mRNA expression levels of TIMP-1 and TIMP-2 were significantly up-regulated in Group A compared with both Group C and Group G. The mRNA expression levels of TIMP-1 and TIMP-2 in Groups A, C, and G were

0.52 ± 0.15 and 0.41 ± 0.09, 0.83 ± 0.38 for TIMP-1, respectively (Fig. 6A). These values were 0.35 ± 0.07, 0.52 ± 0.06, and 2.97 ± 0.77 for TIMP-2, respectively. In addition, the mRNA expression levels of inflammatory cytokines, MCP-1, IL-1β and TNF-α, were all down regulated in group A (MCP-1: 0.31 ± 0.07 in group C, 0.26 ± 0.05 in group G, 0.13 ± 0.02 in group A, IL-1β: 1.93 ± 0.18 in group C, 0.42 ± 0.25 in group G, 0.17 ± 0.18 group A, TNF-α: 2.43 ± 0.17 in group C, 0.76 ± 0.20 in group G, 0.20 ± 0.10 in group A, Fig. 7A). Similarly in CD 68 staining, the number of positive cells in the aortic walls was significantly decreased in group A (87.8 ± 28.1 cells in group C, 46.8 ± 9.5 cells in group G, 17.2 ± 6.4 cells in group A, Fig.7B and 7C). The distribution of MCP-1 were also reduced in group A. Moreover, MMP activity was evaluated by gelatin zymography (Fig.6B) and group A were significantly down-regulated in all the pro MMP-2, MMP-2 and MMP-9 activities (Fig.6C).

DISCUSSION

The current study demonstrated that wrapping an ascorbic acid-saturated gelatin hydrogel sheet around the aorta prevented AAA formation in a rat model. This effect was mediated by an increased level of antioxidant in cooperation with the reinforcement of the aortic wall via preservation of its elastin and collagen.

In vitro release tests demonstrated that an ascorbic acid-containing hydrogel sheet could only generate a concentration of approximately 30 μg/ml, *b*ut i*n vivo* release test showed the concentration could reach around 100μg/ml. The biodegradation of the material *in vitro* is expected to deliver a higher concentration as Konishi et al [13] showed in their experiment of the gelatin hydrogel. They demonstrated that the drug contained in gelatin hydrogel sheet only released 10 to 30% in the *in vitro* test, but with the degradation of gelatin hydrogel *in vivo,* the drug was released more and had effect superior to topical application. The initial release of

ascorbic acid from the sheet that was observed in the release test is assumed to be due to the compound on the surface of the sheet which was not bound to the gelatin hydrogel. Previous report in human showed that the plasma vitamin C concentration can only reach 220 μmol/L, approximately 38μg/ml, with doses of 3 g for 6 times daily.[18] Thus direct delivery of ascorbic acid by gelatin hydrogel can be expected to reach much higher level of concentration than that can be reached with oral supplementing.

Elastin and collagen are important load-bearing components of the aortic wall, and the destruction of these elements leads to aortic dilatation.[19] We conclusively demonstrated that aortic walls that received a topical application of ascorbic acid via a gelatin hydrogel sheet contained larger contents of elastin and collagen and exhibited preserved morphology in the rat AAA model; furthermore, AAA formation was attenuated by this treatment. The elastin and collagen were better preserved by the antioxidant and anti-inflammatory effects of ascorbic acid and may have been newly produced from aortic smooth muscle cells. There are several reports that support this latter hypothesis. Tajima et al[8] previously reported that the production of tropoelastin was induced by the presence of ascorbic acid when aorta-derived smooth muscle cells were cultured. Moreover, Qiao et al[6] reported that the addition of ascorbic acid to the cell culture resulted in procollagen maturation. Ascorbic acid is required in the proline hydroxylation of both collagen and elastin,[20] and hydroxyproline is crucial for collagen and elastin stability, which gives strength to the cellular matrix.

Oxidative stress has been demonstrated to play a significant role in human AAA formation and progression.[2] Our group recently reported that the formation and the development of AAAs were inhibited by the suppression of ROS production.[21] Ascorbic acid is a well-known antioxidant that cannot be produced in the human body. We performed preliminary experiment in our AAA model (N=2) with oral ascorbic acid supplementation of

200 mg daily for 2 weeks, but it could not prevent dilatation of the AAA (dilatation at 4 weeks: 170% and 223%). This result is consistent with Jiang et al [22] previously described in their study that oral supplementation of antioxidant vitamins had minimal effects on prevention of an Angiotensin II-induced AAA formation. However, with our method of delivering ascorbic acid to the target aorta, the DHE staining of aortic cross-section in the present study revealed that ROS expression was apparently suppressed in the outer layer of the aortic wall. Moreover, fewer 8-OHdG positive cells were observed in the aortas exposed to ascorbic acid compared with the other groups. Both findings indicate that gelatin hydrogel sheet successfully released ascorbic acid and had antioxidant effect.

Both smooth muscle cells and infiltrating inflammatory cells produce MMPs. Specifically, MMP-2 and MMP-9 are crucial in the formation and the development of AAAs.[23] As we described previously, elastase and calcium chloride-treated control aortas exhibited markedly increased MMP-2 and MMP-9 activity in whole aorta homogenates.[14] The down-regulations of TIMP-1 and TIMP-2 are reported to result in proteolytic extracellular matrix degradation, a process that also plays key role in aneurysm formation.[24] In the present study, MCP-1 and CD 68staining of AAA wall and mRNA expressions of MCP-1, IL-1β, TNF-α showed ascorbic acid sheet successfully suppressed recruitment of inflammatory cells and inflammatory responses, and subsequently pro MMP-2, MMP-2 and MMP-9 activities were inhibited by treatment with ascorbic acid-impregnated hydrogel sheet; an up-regulation of TIMP-1 and TIMP-2 was also observed in the ascorbic acid treated group.

We performed additional experiment to evaluate the treatment effect of the ascorbate sheet on existing AAA. The animals at 1 week following AAA induction were treated with plain hydrogel sheet (N=2) and ascorbic acid impregnated hydrogel sheet (N=2). The plain sheet could not attenuate the AAA progression (dilatation rate at 1 week 117% and 236%;

dilatation rate at 4 weeks: 234% and 220%) but ascorbic acid sheet did prevent from further dilatation (dilatation reate at 1 weeek: 113% and 133%; dilatation rate at 4 weeks: 134% and 154%). This result shows that ascorbic acid impregnated sheet has possible treatment effect on existing AAA.

Limitations

The limitation of the present study is that the elastase and calcium chloride-induced rat AAA is not completely similar to human AAA as it lacks several prominent features of the human lesions, such as atherosclerosis and intraluminal thrombosis. However, we demonstrated that our AAA model is simple, easy to perform, highly reliable and reproducibly creates a saccular aneurysm.[14]

Conclusions

We demonstrate that the wrapping of ascorbic acid-impregnated gelatin hydrogel sheet has significant protective effects against AAA formation in an elastase and calcium chloride induced rat AAA model with suppressed of inflammatory response and oxidative stress.. Ascorbic acid treatment has possible positive effect on elastin and collagen preservation and production. This treatment also had a protective effect against oxidative stress.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 24592064) from the Japan Society for the Promotion of Science. We thank Atsumi Katsuta for assistance in performing the data analysis.

Disclosure/Duality of interest

None

Figure legends

Fig 1. A: The *in vitro* controlled release test of ascorbic acid over-time.

B: The in vivo controlled release test of ascorbic acid over-time

Fig 2. A: The photographs show treated lesions of abdominal aorta at 4 weeks in each group. Group C, control; Group G, saline-impregnated gelatin hydrogel; Group A, ascorbic acid-impregnated gelatin hydrogel.

B: Dilatation ratio (%) at 4-week and 8-week.

Fig 3. Histologic findings of abdominal aorta cross-sections at 4 weeks after staining with hematoxylin eosin (H&E) and elastic van Gieson (EVG) for the morphologic assessment, and with picrosirius red (PSR) for the collagen content assessment. The upper rows are at the original magnification (40X; Bar, 300μm), and the lower panels are at the original magnification (400X; Bar, 100μm). Native, normal rats; Group C, control; Group G, saline-impregnated gelatin hydrogel; Group A, ascorbic acid-impregnated gelatin hydrogel.

Fig 4. The quantification of elastin (A) and collagen (B) at 4 weeks. All of the data are expressed as the mean ± standard deviation for 6 rats in each group. NS, Not significant. Native, normal rats; Group C, control; Group G, saline-impregnated gelatin hydrogel; Group A, ascorbic acid-impregnated gelatin hydrogel.

Fig 5. The assessment of oxidative stress in the aortic wall at day 7.

A, Abdominal aorta cross-sections at 1 week after staining with dihydroethidium (DHE) for the in situ imaging of superoxide levels and 8-hydroxydeoxyguanosine (8-OHdG) staining for the assessment of oxidative DNA damage. Star, inner aortic lumen. Native, normal rats; Group C, control; Group G, saline-impregnated gelatin hydrogel; Group A, ascorbic acid-impregnated gelatin hydrogel.

B, The dihydroethidium (DHE) count was semiquantified and expressed relative to values that were obtained for native rat. All of the data are expressed as the mean ± standard deviation for 6 rats (average of randomly selected five fields) in each group. C, 8-OHdG positive cell count in a 500 μm by 380 μm rectangle of the aortic wall. All of the data are expressed as the mean ± standard deviation for 6 rats (average of randomly selected five fields) in each group.

Fig 6. A: Messenger RNA (mRNA) expression of matrix metalloprotenases, given as a ratio of glyceraldehyde-3-phosphatedehydrogenase GAPDH expression in the abdominal aorta at 1 week. All of the data are expressed as the mean ± standard deviation for 6 rats in each group. MMP: matrix metalloproteinase, TIMP: tissue inhibitors of metalloproteinase. NS, Not significant. Native, normal rats; Group C, control; Group G, saline-impregnated gelatin hydrogel; Group A, ascorbic acid-impregnated gelatin hydrogel. * P< .05, **P< .01, ***P< .001

B: Gelatin zymography documents gelatinolytic activities of pro MMP-2, MMP-2 and MMP-9

C: Densitometric analysis of the MMP activities.

Fig. 7 A: Messenger RNA (mRNA) expressions of inflammatory cytokines, given as a ratio of glyceraldehyde-3-phosphatedehydrogenase GAPDH expression in the abdominal aorta at 1 week. All of the data are expressed as the mean ± standard deviation for 6 rats in each group. MCP-1, monocyte chemotactic protein-1; IL-1β, Interleukin-1β; tissue necrosis factor-α, TNF-α. NS, not significant * P< .05, **P< .01, ***P< .001

B: Cluster differentiation (CD) 68 positive cell count in a 500 μm by 380 μm rectangle of the aortic wall. All of the data are expressed as the mean ± standard deviation for 6 rats (average of randomly selected five fields) in each group.

C: CD68 and monocyte chemotactic protein (MCP)-1 staining for assessment of macrophage recruitment

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Fig.1

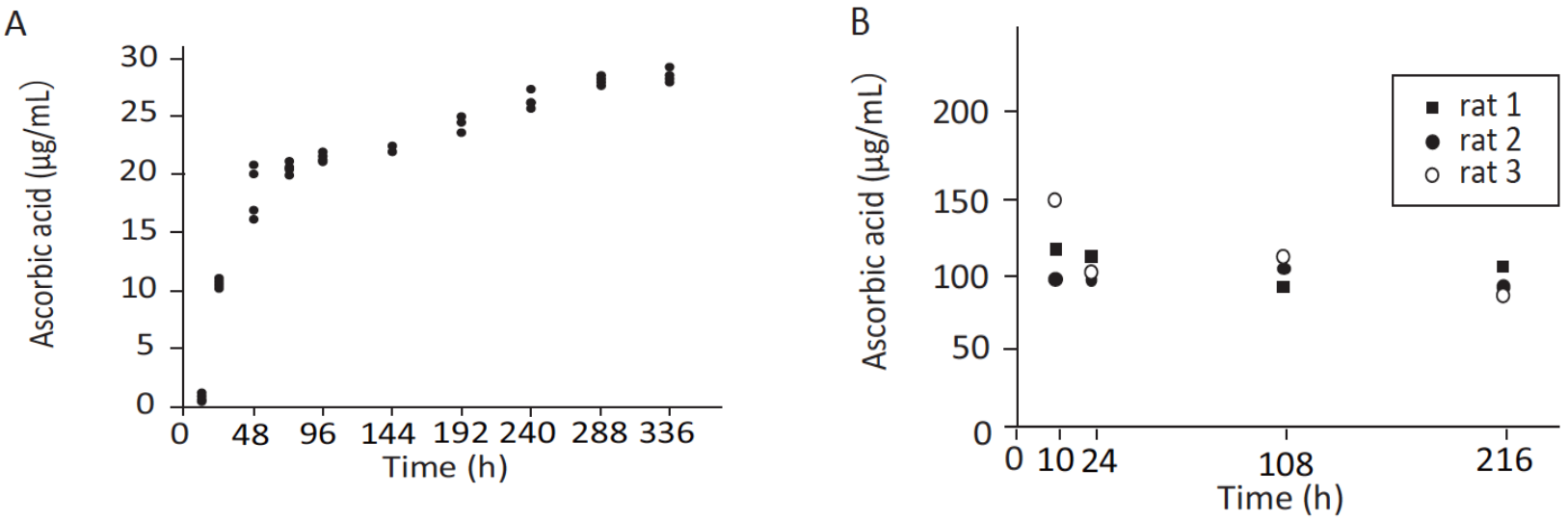
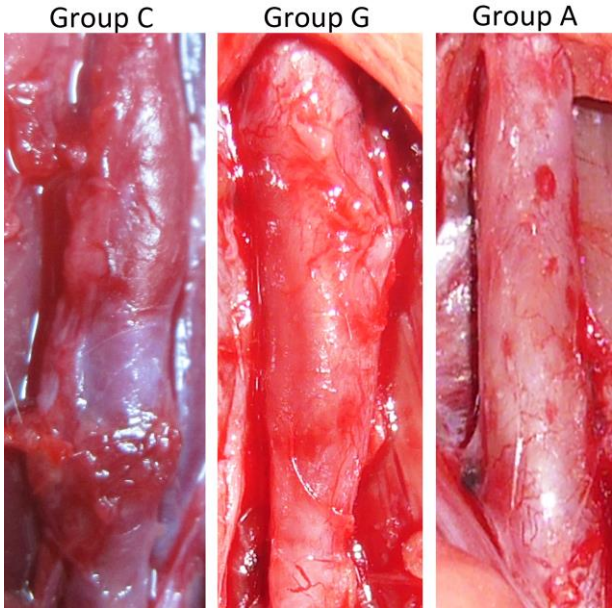
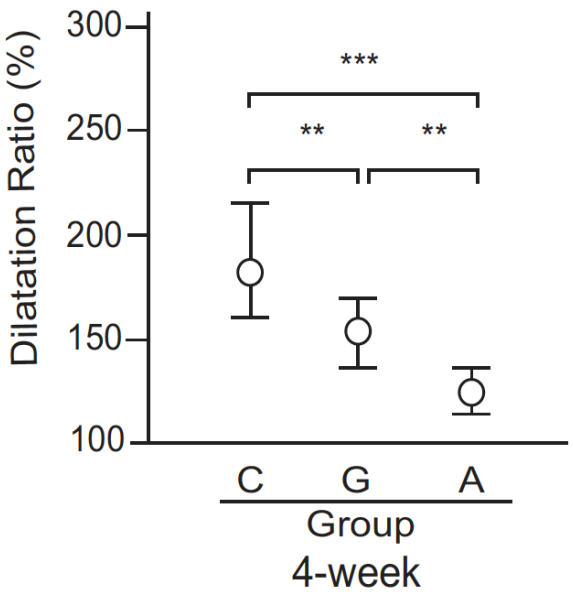

A
Ascorbic acid (μg/mL)
30
25
20
15
10
5
0
0
48
96
144
192
240
288
336
Time (h)
B
Ascorbic acid (μg/mL)
200
150
100
50
0
0
10
24
108
216
Time (h)
rat 1
rat 2
rat 3

Fig. 2

A


Group C
Group G
Group A

B


Dilatation Ratio (%)
300
250
200
150
100
***
**
**
C
G
A
Group
4-week

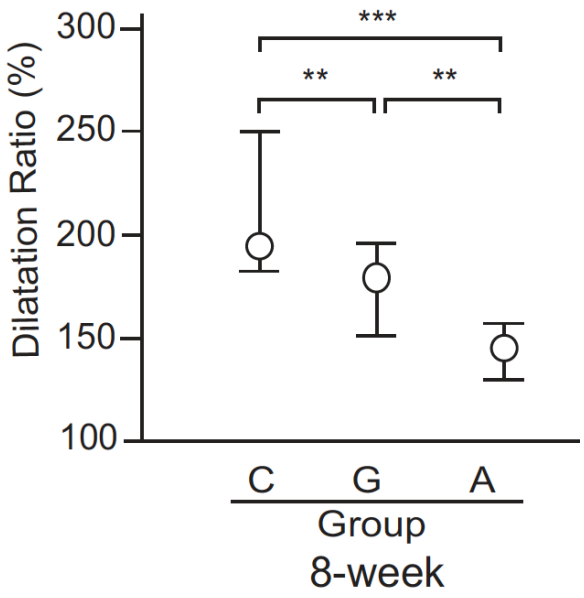

Dilatation Ratio (%)
300
250
200
150
100
***
**
**
C
G
A
Group
8-week

Fig.3

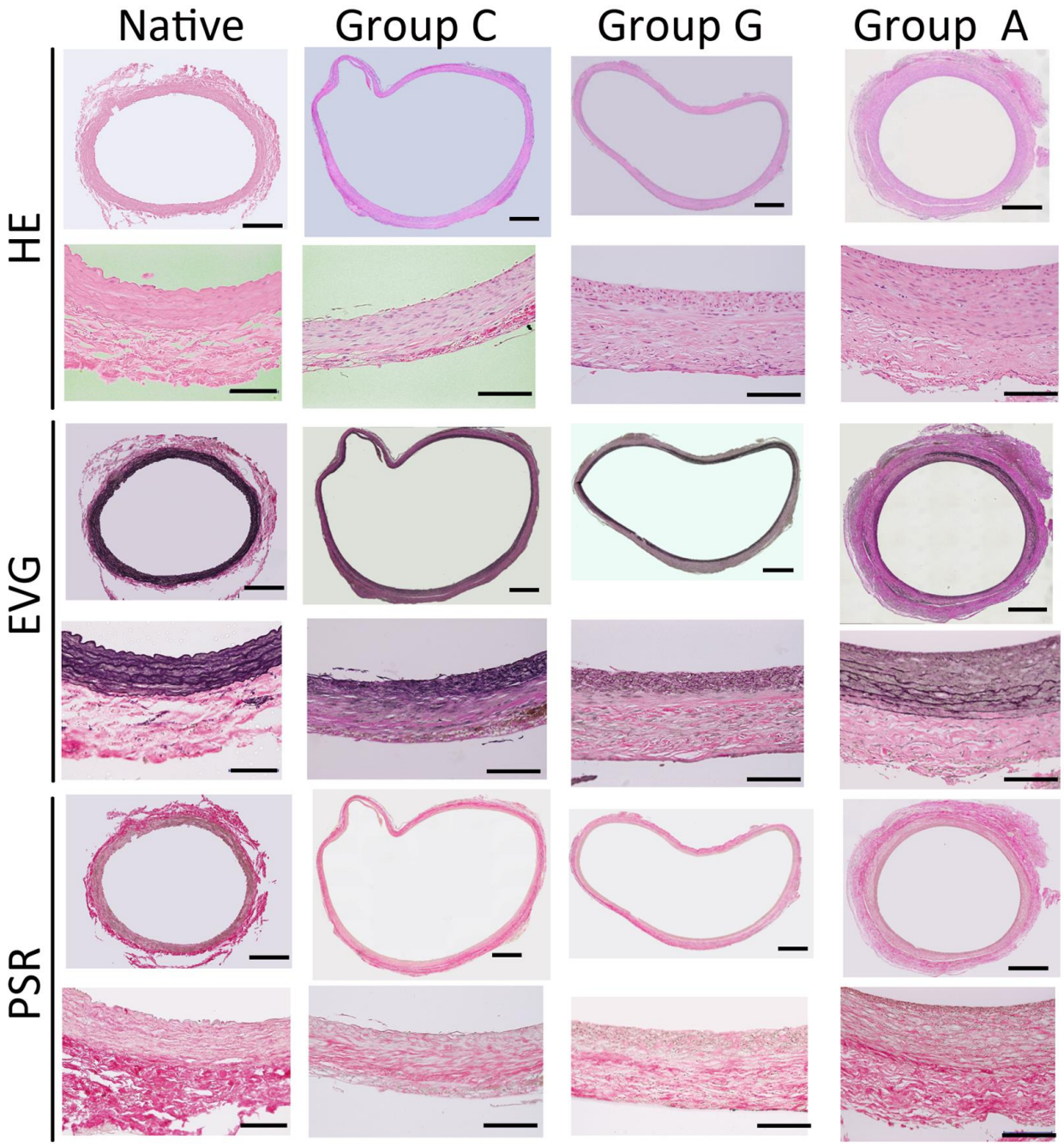


Fig.4

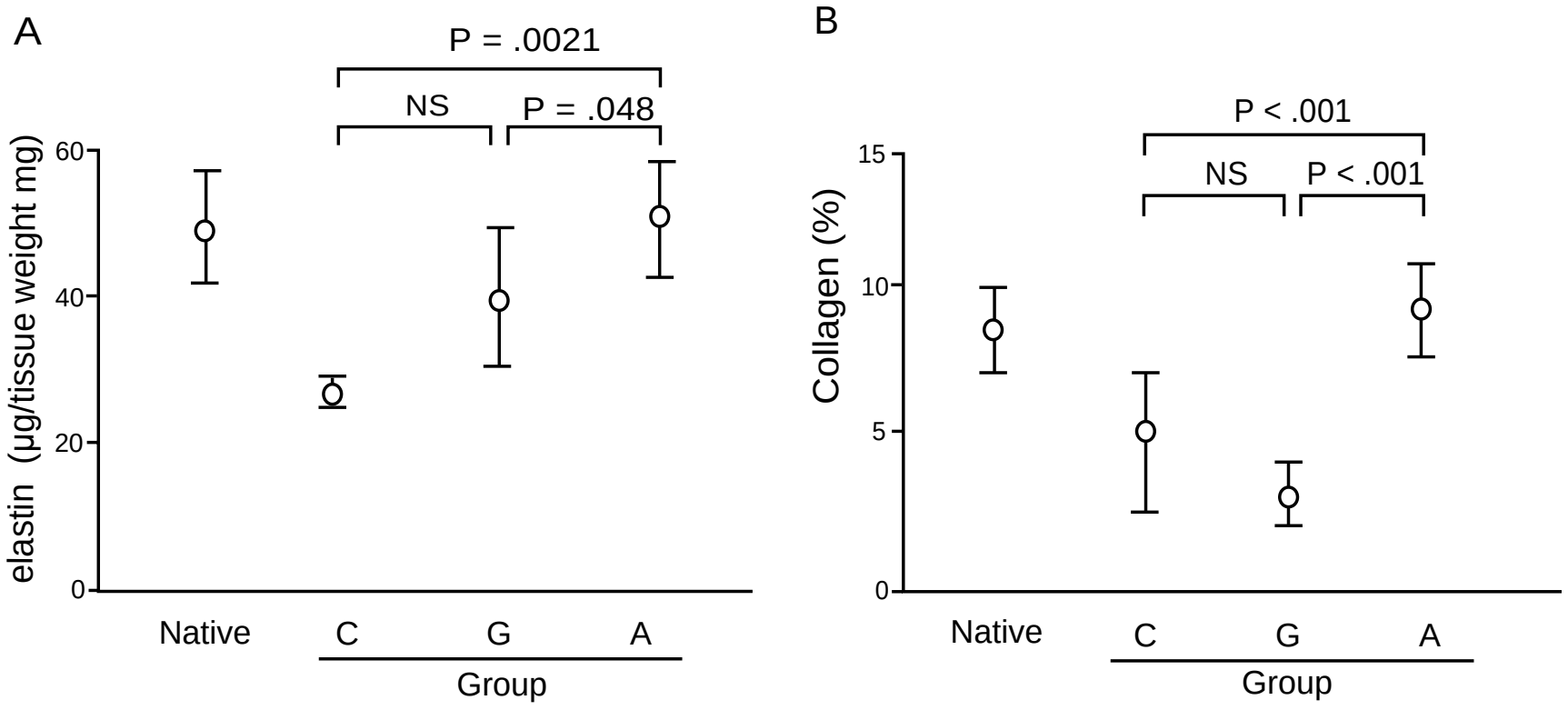

A
P = .0021
NS
P = .048
elastin (μg/tissue weight mg)
60
40
20
0
Native
C
G
A
Group
B
P < .001
NS
P < .001
Collagen (%)
15
10
5
0
Native
C
G
A
Group

Fig.5

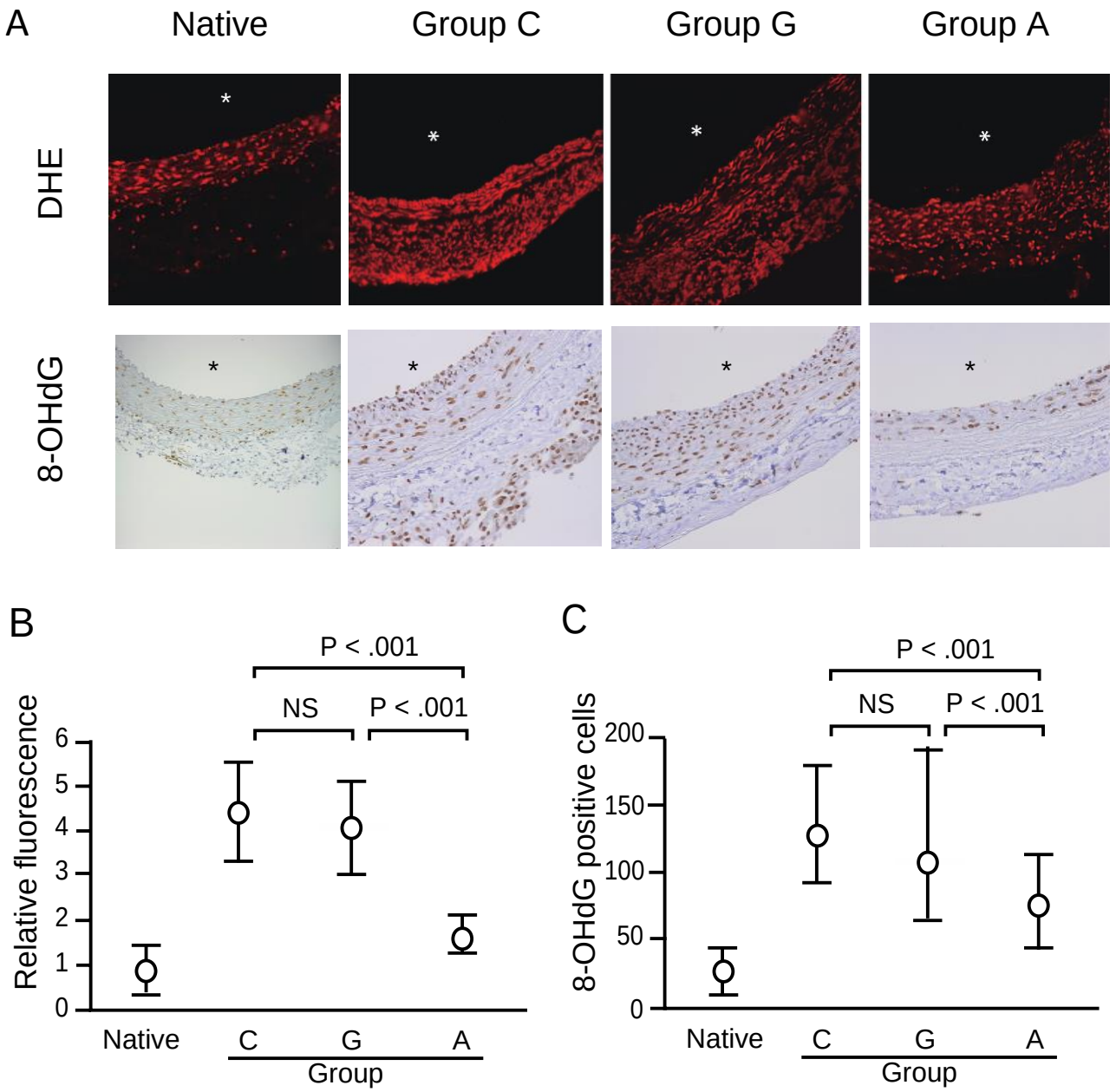

A
Native
Group C
Group G
Group A
DHE
8-OHdG
B
P < .001
NS
P < .001
Relative fluorescence
0
1
2
3
4
5
6
Native
C
G
A
Group
C
P < .001
NS
P < .001
8-OHdG positive cells
0
50
100
150
200
Native
C
G
A
Group

Fig.6

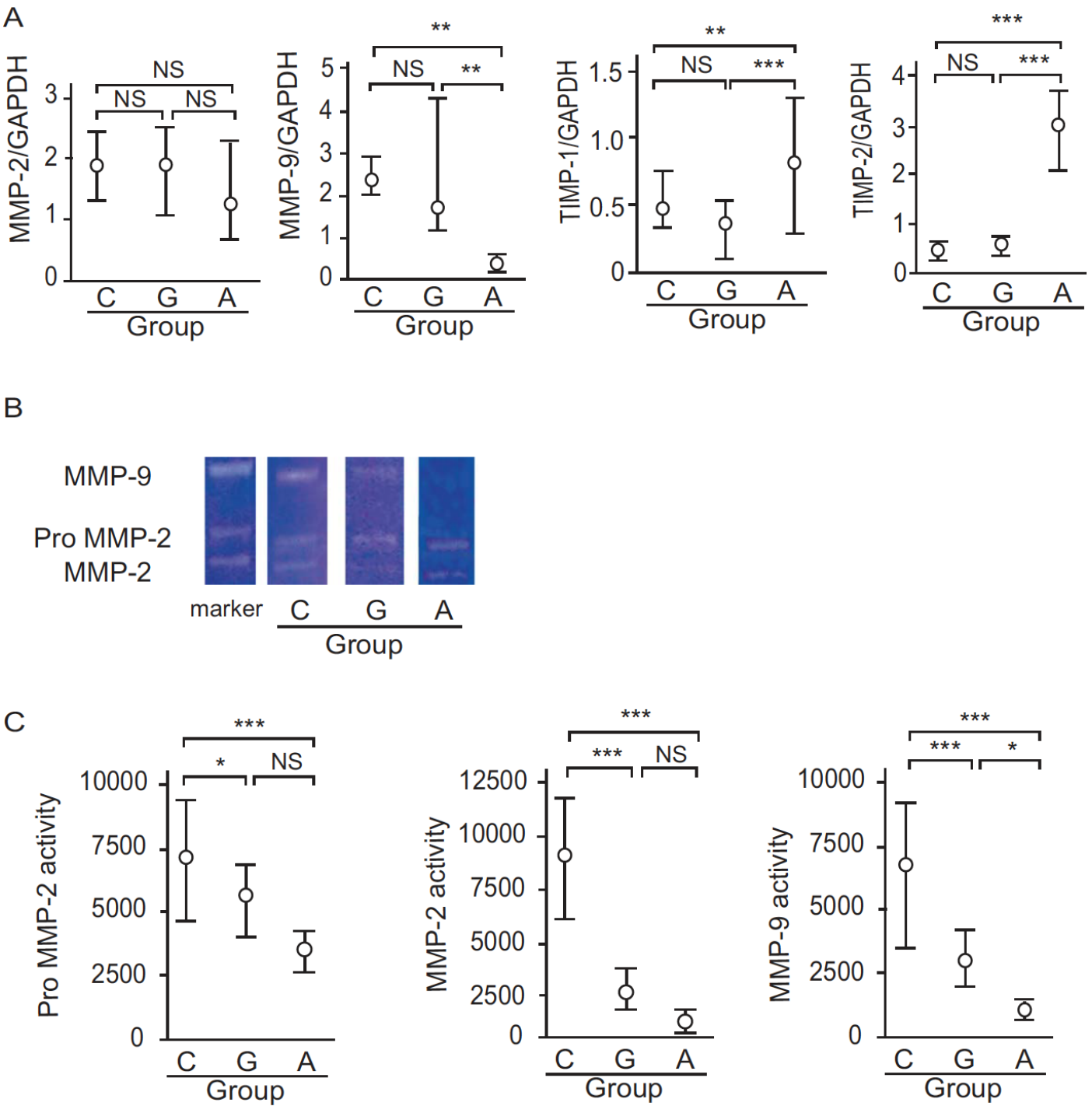
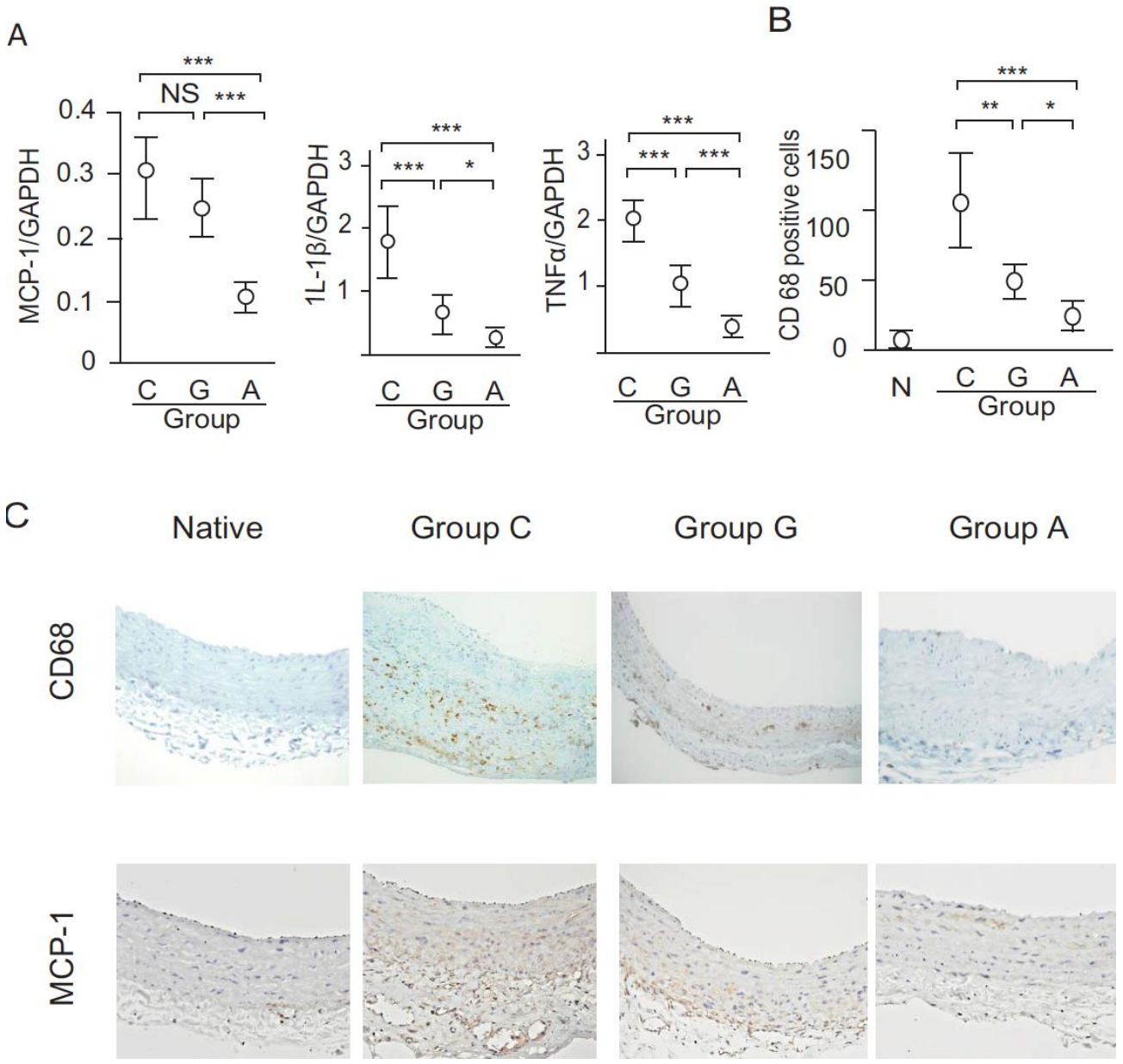

A
MMP-2/GAPDH
NS
NS
NS
C
G
A
Group
MMP-9/GAPDH
**
NS
**
Group
TIMP-1/GAPDH
**
NS
***
Group
TIMP-2/GAPDH
***
NS
***
Group
B
MMP-9
Pro MMP-2
MMP-2
marker
C
G
A
Group
C
Pro MMP-2 activity
***
*
NS
Group
MMP-2 activity
***
***
NS
Group
MMP-9 activity
***
***
*
Group

Fig. 7


A
B
C
MCP-1/GAPDH
1L-1β/GAPDH
TNFα/GAPDH
CD 68 positive cells
Group
C G A
N
NS
***
**
*
Native
Group C
Group G
Group A
CD68
MCP-1