The consequences of subarachnoid hemorrhage (SAH) after cerebral aneurysm rupture are devastating, with mortality rates as high as 50% and the majority of survivors left with moderate to severe disability. Cerebral vasospasm causes cerebral ischemia and is a major contributor to the high morbidity and mortality rates associated with SAH. The pathogenesis of cerebral vasospasm, however, has not been fully clarified, and potential preventative therapies for this phenomenon have not been established.

Oxyhemoglobin derived from a subarachnoid clot and the free radicals subsequently generated have been considered important causes of cerebral vasospasm. These radicals, by means of a scavenging effect, may be related to cerebral vasospasm by suppressing nitric oxide (NO) synthase (eNOS). So, apple polyphenol might be effective in the prevention of vasospasm due to an abundant content of procyanidins, which exhibit strong radical scavenging effects, and the ability to suppress ox-LDL and LOX-1. The purposes of this study were to investigate changes in levels of ox-LDL and LOX-1 after SAH and whether administering apple polyphenol can modify cerebral vasospasm.

Object. Cerebral vasospasm after subarachnoid hemorrhage (SAH) is a serious complication. Free radicals derived from subarachnoid clotting are recognized to play an important role. Oxidized low-density lipoprotein (ox-LDL) and lectin-like oxidized LDL receptor-1 (LOX-1) have been shown to be related to the pathogenesis of atherosclerosis and may increase in cerebral arteries after SAH, due to the action of free radicals derived from a subarachnoid clot. These molecules may also affect the pathogenesis of vasospasm, generating intracellular reactive oxygen species and downregulating the expression of endothelial NO synthase (eNOS). So, apple polyphenol might be effective in the prevention of vasospasm due to an abundant content of procyanidins, which exhibit strong radical scavenging effects, and the ability to suppress ox-LDL and LOX-1. The purposes of this study were to investigate changes in levels of ox-LDL and LOX-1 after SAH and whether administering apple polyphenol can modify cerebral vasospasm.

Methods. Forty Japanese white rabbits were assigned randomly to 4 groups: an SAH group (n = 10); a sham-operation group (n = 10), which underwent intracisternal saline injection; a low-dose polyphenol group (n = 10) with SAH and oral administration of apple polyphenol at 10 mg/kg per day from Day 0 to Day 3; and a high-dose polyphenol group (n = 10) with SAH and oral administration of apple polyphenol at 50 mg/kg per day. At Day 4, the basilar artery and brain was excised from each rabbit. The degree of cerebral vasospasm was evaluated by measuring the cross-sectional area of each basilar artery, and the expression of ox-LDL, LOX-1, and eNOS was examined for each basilar artery by immunohistochemical staining and reverse transcriptase polymerase chain reaction. In addition, neuronal apoptosis in the cerebral cortex was evaluated by TUNEL.

Results. Compared with the sham group, the expression of ox-LDL and LOX-1 in the basilar arterial wall was significantly increased in the SAH group, the expression of eNOS was significantly decreased, and the cross-sectional area of basilar artery was significantly decreased. Compared with the SAH group, the cross-sectional area of basilar artery was increased in the polyphenol groups, together with the decreased expression of ox-LDL and LOX-1 and the increased expression of eNOS. In the high-dose polyphenol group, those changes were statistically significant compared with the SAH group. In the low-dose polyphenol group, those changes were smaller than in the high-dose polyphenol group. No apoptosis and no changes were seen in the cerebral cortex in all groups.

Conclusions. This is the first study suggesting that ox-LDL and LOX-1 increase due to SAH and that they may play a role in the pathogenesis of vasospasm. It is assumed that procyanidins in apple polyphenol may inhibit a vicious cycle of ox-LDL, LOX-1, and ROS in a dose-dependent manner. Apple polyphenol is a candidate for preventive treatment of cerebral vasospasm.

Key Words • subarachnoid hemorrhage • cerebral vasospasm • free radical • oxidized low-density lipoprotein • lectin-like oxidized LDL receptor-1 • polyphenol • vascular disorders

Abbreviations used in this paper: eNOS = endothelial NO synthase; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; LDL = low-density lipoprotein; LOX-1 = lectin-like oxidized LDL receptor-1; ox-LDL = oxidized LDL; NIH = National Institutes of Health; NO = nitric oxide; PBS = phosphate-buffered saline; ROS = reactive oxygen species; RT-PCR = reverse transcriptase polymerase chain reaction; SAH = subarachnoid hemorrhage; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

THE con...
ide (NO), downregulating endothelial NO synthase (eNOS), stimulating the release of vasoconstricting endothelin and prostaglandins from endothelial cells, and by activating lipid peroxidation to invoke various intracellular signaling transduction pathways linked to the pathogenesis of cerebral vasospasm.

With recent advances in angiology and vascular research, oxidized low-density lipoprotein (ox-LDL) and its receptor, lectin-like ox-LDL receptor-1 (LOX-1), which are among many indicators of radical reactions or oxidative stress, have been attracting attention. Low-density lipoprotein is converted to ox-LDL through oxidative modification by free radicals, and ox-LDL upregulates LOX-1. Ox-LDL and LOX-1 have been revealed to play important roles in the pathogenesis of atherosclerosis by mediating various reactions and processes.

It has been reported that, in turn, ox-LDL binding to LOX-1 increases intracellular reactive oxygen species (ROS), leading to a reduction in nitric oxide (NO) availability and downregulation of eNOS and initiating the inhibition of vasodilatation. Considering these actions of ox-LDL and LOX-1, ox-LDL and LOX-1 could thus be expected to play an important role in the pathogenesis of cerebral vasospasm; however, no report has investigated the relationship between ox-LDL, LOX-1, and cerebral vasospasm. One purpose of this study was to clarify this relationship by investigating whether ox-LDL and LOX-1 in the cerebral arterial wall, accompanied by eNOS, were changed after SAH.

We note here that, in terms of preventing cerebral vasospasm, several free radical scavengers had been tried; however, their preventive effects have not been established, and there are no commercially available free radical scavengers for cerebral vasospasm.

In contrast to synthetic free radical scavengers, polyphenol is a natural substance that exhibits potential radical scavenging effects without major harmful side effects. Of the polyphenols, procyanidin, which is a oligomer of epicatechin, features strong radical scavenging activity due to its many hydroxyl groups, has significant capacity to protect LDL from oxidation, and is the only molecule that has been found to exhibit an action directly antagonistic to LOX-1. Procyanidin content is higher in apples than in other commonly consumed foods, and procyanidin is a main component of polyphenols extracted from apples. A second purpose of this study was to investigate whether administration of apple polyphenol contributes to preventing cerebral vasospasm through scavenging free radicals and suppressing ox-LDL and LOX-1. In addition, attention has recently focused on early brain injury, including neuronal apoptosis, as a factor contributing to poor outcome after SAH, which is reportedly ameliorated by suppressing oxidative stress. We therefore also tried to elucidate whether early brain injury could be modified by the administration of apple polyphenol by examining neuronal apoptosis of the cerebral cortex.

To address both of these issues, we conducted an experimental study using a rabbit SAH model.

Methods

Animal Population

All experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH) and approved by the Hirosaki University Animal Research Committee.

All Japanese white rabbits used in the present study were purchased from the Kitayama Labes Co., Ltd. They were maintained on the standard pellet diet at the Institute for Animal Experiments of Hirosaki University School of Medicine. The temperature in both the feeding and operation rooms was maintained at approximately 25°C.

Animal Experimental Design

Forty 12-week-old female Japanese white rabbits weighing from 2.5 kg to 2.99 kg were assigned randomly to 4 groups: 1) SAH group, SAH was produced as described below and animals were killed on Day 4 (n = 10); 2) sham group, physiological saline instead of blood was injected into the cisterna magna twice and animals were killed on Day 4 (n = 10); 3) low-dose polyphenol group, after production of SAH, animals were administered apple polyphenol at a dosage of 10 mg/kg orally once a day from Day 0 to Day 3 and were killed on day 4 (n = 10); and 4) high-dose polyphenol group, after production of SAH, animals were administered apple polyphenol at a dosage of 50 mg/kg orally once a day from Day 0 to Day 3 and were killed on Day 4 (n = 10).

Perfusion-fixation was performed under deep anesthesia in 5 rabbits in each group for histological and immunohistochemical evaluation of the basilar artery and the cerebral cortex. The other 5 rabbits in each group were killed without perfusion-fixation, and each basilar artery was immediately excised and stored at −80°C for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Induction of Experimental SAH

Experimental SAH was produced on Day 0 and on Day 2 according to the double-hemorrhage method. The rabbits were anesthetized by an intravenous injection of pentobarbital (30 mg/kg) and an intramuscular injection of ketamine (20 mg/kg).

After anesthesia, under spontaneous breathing, a 23-gauge butterfly needle was inserted percutaneously into the cisterna magna. After aspiration of 1.5 mL of cerebrospinal fluid, the same amount of nonheparinized arterial blood from the femoral artery was slowly injected into the cisterna magna for 1 minute under aseptic technique. Rabbits were then placed in a 30° head-down position for 30 minutes. After recovery from anesthesia, they were returned to the feeding room. Forty-eight hours afterward, the second experimental SAH was produced in the same manner as the first.

In the sham group, the same technique was applied, with injection of sterile saline instead of blood.

Administration of Apple Polyphenol

In the low-dose and the high-dose polyphenol groups,
apple polyphenol (10 mg/kg/day or 50 mg/kg/day, respectively) was administered orally using a nasogastric tube once per day for 4 days from Day 0 to Day 3.

In this study, we used commercially available apple polyphenol (Asahi Food and Healthcare Co. Ltd), which contains 63.8% procyanidin, has been used widely as a dietary supplement or as an antioxidant food additive for various processed foods in Japan, and was generally recognized as safe in 2004 in accordance with regulations set by the US Food and Drug Administration.

Perfusion-Fixation

Perfusion-fixation was then performed on Day 4 under deep anesthesia induced by means of an intravenous injection of high-dose pentobarbital (300 mg/kg) and an intramuscular injection of ketamine (100 mg/kg). The thorax was opened, and a cannula was immediately inserted into the ascending aorta via the left ventricle. Subsequently, the descending aorta was clamped, and the right atrium was opened. Perfusion was begun with 500 ml of heparinized physiological saline (5000 U/500 ml) at 37°C, followed by 500 ml of phosphate-buffered 4% paraformaldehyde (pH 7.4) under a perfusion pressure of 75 mm Hg. Finally, the whole brain was excised with the basilar artery, with care taken to avoid stretching or injuring the basilar artery.

Measurement of Basilar Artery Cross-Sectional Area

The degree of cerebral vasospasm was evaluated by measuring the cross-sectional area of the basilar artery lumen. The formalin-fixed and paraffin-embedded basilar artery sections (6 μm thick) were deparaffinized, hydrated, washed, and stained with H & E. Micrographs of the basilar arteries were analyzed by using ImageJ version 1.45 (NIH). Cross-sectional areas of basilar arteries were calculated from the perimeter of the luminal border. For each vessel, 3 sequential sections (midpoint of proximal, middle, and distal) were taken, measured, and averaged. The mean ± SEM value obtained for each artery was used as the final value for a particular vessel.

Immunohistochemical Analysis of the Basilar Artery

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections to determine the immunoreactivity of ox-LDL, LOX-1, and eNOS. Sections were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Sections were placed in EDTA buffer (pH 9.0), heated in a microwave oven for 15 minutes, and then cooled at room temperature for 20 minutes and rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% H2O2 for 5 minutes, followed by a brief rinse in distilled water and a 15-minute wash in PBS. Nonspecific protein binding was blocked by 5% horse serum. Sections were incubated with primary anti–Cu2+-oxidized LDL antibody (1:200 dilution, Merck), primary anti–LOX-1 antibody (1:200 dilution, Abcam), or primary anti–eNOS antibody (1:200 dilution, BD Biosciences) for 60 minutes at room temperature, followed by a 15-minute wash in PBS. Sections were incubated with goat anti–rabbit IgG (1:500 dilution) for 60 minutes at room temperature. Diaminobenzidine was used as a chromogen, and counterstaining was performed with hematoxylin.

To thoroughly evaluate the expression of ox-LDL, LOX-1, and eNOS, the basilar artery wall was subdivided into 4 subregions: endothelium, subendothelium, smooth muscle layer, and adventitia. The intensity of immunohistochemical staining was examined in each subregion and evaluated using a semiquantitative scoring system by which the intensity was scored from 0 to 2 (0, none; 1, slight; 2, intense). Each specimen was evaluated in a blinded fashion by 3 independent observers (A.M., N.S., and K.A.).

RT-PCR Analysis of the Basilar Artery

Total RNA was isolated from rabbit basilar artery tissues using a Pure Link RNA Mini Kit (Ambion) after the tissues were homogenized with a Biomasher II (Nippi). Then 1.0 μg of total RNA was reverse-transcribed into cDNA with oligo-dT and Prime Script RT-PCR Kit (TaKaRa) at 42°C for 50 minutes. The reverse-transcribed material (2 μl) was amplified with Prime Script RT-PCR Kit (TaKaRa) by using a primer pair specific to rabbit LOX-1 cDNA (sense primer, 5′-TTG ACCCTACCTACATCTGTACC-3′; antisense primer, 5′-CAAGAGCCACCGAGCAATGA-3′, Fasmac). The PCR profile was set at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute for 40 cycles. The primer pair for rabbit eNOS (sense primer, 5′-CAGTTG CCAAATGCTGCTGAAATTTG-3′; antisense primer, 5′-TAAAGGTCTTCTCTCCCTGGTATGCC-3′, Fasmac), and the PCR profile were set at 94°C for 40 seconds, 58°C for 1 minute, and 70°C for 1 minute for 30 cycles. Also, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was amplified as a reference for quantification of LOX-1 mRNA and eNOS mRNA using a primer pair specific to rabbit GAPDH (sense primer, 5′-GAG CTGAGCCGAAAACCTCAC-3′; antisense primer, 5′-GGTTGCGGATGAAACTGTG-3′, Fasmac) under the same reaction conditions as LOX-1 mRNA for 30 cycles. The amplified transcripts were visualized on 1% agarose gels with the use of ethidium bromide. Specific amplification products of the expected size (386 bp for LOX-1, 485 bp for eNOS, and 476 bp for GAPDH) were observed. Relative intensities of the bands of interest were analyzed using Image Lab software Version 3.0 (Bio-Rad). The ratios of LOX-1 mRNA and eNOS mRNA to GAPDH mRNA were quantified.

Assessment of Brain Damage

Temporal lobes excised after perfusion-fixation were embedded in paraffin, and 4-μm-thick coronal sections were examined to detect neuronal apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

A Wako Apoptosis in situ Detection Kit was used according to the manufacturer's instructions. Paraffin-embedded sections were mounted on positively charged slides, deparaffinized, rehydrated, and washed thoroughly with distilled water. The tissues were digested with
protease at 37°C for 5 minutes. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide solution in PBS at room temperature for 5 minutes. The sections were then incubated with terminal deoxynucleotidyl transferase at 37°C for 10 minutes, which adds the digoxigenin-conjugated dUTP to the 3′-OH ends of fragmented DNA. Anti-digoxigenin antibody horseradish peroxidase conjugate was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counterstained slightly with methyl green solution.

**Statistical Analysis**

All data are expressed as means ± SEM. In the analysis of the area of basilar artery and RT-PCR, means of different groups were compared with ANOVA followed by Tukey’s multiple comparisons test. In the analysis of immunohistochemical staining, means of different groups were compared with the Kruskal-Wallis test followed by the Scheffé test. A p value less than 0.05 was considered to indicate statistical significance.

**Results**

**Evaluation of Cerebral Vasospasm and Vascular Patency**

The cross sections of the basilar artery in each group, determined by staining with H & E and observation under the light microscope, are shown in Fig. 1A–D.

The mean areas of the basilar artery in the SAH group, in the sham group, in the low-dose polyphenol group, and in the high-dose polyphenol group were 0.19 ± 0.03 mm², 0.36 ± 0.03 mm², 0.25 ± 0.03 mm², and 0.32 ± 0.01 mm², respectively. There were significant differences between the SAH group and the sham group and between the SAH group and the high-dose polyphenol group (Fig. 1E). In the low-dose polyphenol group, the mean area of the basilar artery was greater than in the SAH group, but the difference was not statistically significant.

**Immunohistochemical Analysis of ox-LDL, LOX-1, and eNOS in the Basilar Artery**

Staining intensity scores for ox-LDL, LOX-1, and eNOS in each subregion are shown in Table 1.

In the SAH group, staining of ox-LDL was seen throughout the entire layer of the arterial wall and was particularly strong at the subendothelial layer and at the adventitia (Fig. 2A). In the sham group, staining was absent or fainter throughout the entire layer of the arterial wall (Fig. 2B). In the low-dose polyphenol group as compared with the SAH group, staining of ox-LDL at the smooth muscle layer was markedly reduced, but staining at the subendothelial layer and adventitia remained (Fig. 2C). In the high-dose polyphenol group, staining was faint throughout the entire layer compared with the SAH group (Fig. 2D), and staining intensity scores at the subendothelium were significantly lower than in the SAH group (Table 1).

LOX-1 was intensely stained throughout the entire layer of the arterial wall in the SAH group (Fig. 2E). In comparison with the SAH group, staining of LOX-1 was significantly fainter throughout the entire layer of the arterial wall in the other 3 groups (Fig. 2F–H; Table 1), and the degree of staining reduction was greater in the high-dose polyphenol group than in the low-dose polyphenol group (Table 1).

In immunohistochemical analysis of eNOS, faint staining at the endothelium was seen in the SAH group (Fig. 2I). In the sham group (Fig. 2J) and in the high-dose polyphenol group (Fig. 2L), the staining intensity score for eNOS was significantly increased at the endothelium compared with the SAH group (Table 1). In the low-dose polyphenol group, eNOS staining was evident at the endothelium, but it was faint (Fig. 2K).

**RT-PCR Analysis of LOX-1 and eNOS mRNA of the Basilar Artery**

The ratio for LOX-1 mRNA as compared with GAPDH mRNA in the SAH group (expressed as x:1) was 6.84 ± 2.70, which was significantly larger than that of the sham group (1.99 ± 1.16), that of the low-dose polyphenol group (3.10 ± 0.54), and that of the high-dose polyphenol group (2.23 ± 0.80) (Fig. 3). The ratio for eNOS mRNA to GAPDH mRNA in the SAH group was 0.26 ± 0.15, which was significantly smaller than that of the sham group (1.15 ± 0.45) and the high-dose polyphenol group (0.59 ± 0.37) (Fig. 4). The ratio in the low-dose polyphenol group (0.50 ± 0.09) was greater than the ratio in the SAH group, but the difference was not statistically significant.

**Evaluation of Brain Damage**

Hematoxylin and eosin staining of the temporal lobe cortex showed no remarkable differences among the groups (Fig. 5E–H). No TUNEL-positive cell was seen in any area of brain in any of the groups, including the SAH group (Fig. 5A–D).

**Discussion**

The results of this study permit speculation about the mechanisms and roles of ox-LDL and LOX-1 in the pathogenesis of cerebral vasospasm, as per Fig. 6.

In this study, immunohistochemical analysis showed ox-LDL was intensely stained, especially at the subendothelial layer and at the adventitia in the SAH group. The source of LDL at the subendothelial layer might be circulating blood. The blood-arterial wall barrier in the major cerebral arteries is disrupted by SAH, which can allow penetration of plasma components into the vessel wall[30,36,40] (Fig. 6A). Free radicals and ROS largely generated at the subarachnoid space after SAH[23,24,43] could also increase the permeability of vessel endothelium and permit LDL infiltration into subendothelium[46] (Fig. 6A). On the other hand, the source of LDL at the adventitia might be subarachnoid blood existing around the major arteries (Fig. 6A).

Ox-LDL is known to trigger upregulation of LOX-1 in mRNA and thereby increase the level of resulting protein[45] (Fig. 6A), which was confirmed in the SAH group in this study. It has been indicated that ox-LDL and LOX-
Oxidized-LDL and LOX-1 in cerebral vasospasm after SAH

1 are easily combined and increase intracellular ROS, which can, in turn, oxidize LDL (Fig. 6B). LOX-1, activated at the endothelial cells by the binding of ox-LDL, could increase the permeability of vessel endothelium and thus facilitate infiltration of LDL from circulating blood into the arterial wall (Fig. 6B). Therefore, ox-LDL, LOX-1, and ROS may participate in a vicious cycle, which could result in a dramatic, persistent increase in ROS, which could influence the artery adversely for a considerable time.

Increased levels of ox-LDL and LOX-1 are assumed to be involved in the pathogenesis of cerebral vasospasm.

Fig. 1. Histological findings for the basilar artery. A–D: Photomicrographs of representative sections from the SAH group, the sham group, the low-dose polyphenol group, and the high-dose polyphenol group are shown. E: Evaluation of cross-sectional area for the basilar artery revealed a statistically significant (*) decrease in the SAH group in comparison with the sham group. The area in the SAH group was also less than in both polyphenol groups, but the difference was statistically significant only at the high polyphenol dosage. Original magnification ×100. Bar = 200 μm.
TABLE 1: Staining intensity scoring of immunohistochemical staining*

<table>
<thead>
<tr>
<th>Parameter &amp; Subregion</th>
<th>SAH</th>
<th>Sham</th>
<th>Low-Dose</th>
<th>High-Dose</th>
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</thead>
<tbody>
<tr>
<td><strong>ox-LDL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endothelium</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>subendothelium</td>
<td>1.07 ± 0.18</td>
<td>0.20 ± 0.11†</td>
<td>0.80 ± 0.20</td>
<td>0.33 ± 0.13‡</td>
</tr>
<tr>
<td>smooth muscle layer</td>
<td>0.40 ± 0.13</td>
<td>0.07 ± 0.07</td>
<td>0.20 ± 0.11</td>
<td>0.20 ± 0.11</td>
</tr>
<tr>
<td>adventitia</td>
<td>1.53 ± 0.14§</td>
<td>0.47 ± 0.17</td>
<td>1.40 ± 0.13§</td>
<td>1.33 ± 0.13§</td>
</tr>
<tr>
<td><strong>LOX-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endothelium</td>
<td>1.60 ± 0.14</td>
<td>0.33 ± 0.12†</td>
<td>0.80 ± 0.20†</td>
<td>0.40 ± 0.13†</td>
</tr>
<tr>
<td>subendothelium</td>
<td>1.80 ± 0.11</td>
<td>0.33 ± 0.13†</td>
<td>0.87 ± 0.09†</td>
<td>0.40 ± 0.13†</td>
</tr>
<tr>
<td>smooth muscle layer</td>
<td>1.80 ± 0.11</td>
<td>0.40 ± 0.13†</td>
<td>0.87 ± 0.09†</td>
<td>0.60 ± 0.13†</td>
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<td>0.47 ± 0.17</td>
<td>1.33 ± 0.13§</td>
<td>1.33 ± 0.13§</td>
</tr>
<tr>
<td><strong>eNOS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endothelium</td>
<td>0.13 ± 0.09</td>
<td>1.47 ± 0.13†</td>
<td>0.47 ± 0.13§</td>
<td>1.13 ± 0.19†</td>
</tr>
<tr>
<td>subendothelium</td>
<td>—</td>
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<td>smooth muscle layer</td>
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<tr>
<td>adventitia</td>
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</table>

* Values are given as mean ± SEM. eNOS = endothelial nitric oxide synthase; LOX-1 = lectin-like oxidized low-density lipoprotein receptor-1; Ox-LDL = oxidized low-density lipoprotein; — = absent or so low as to not be quantifiable.
† p < 0.01 vs SAH group.
‡ p < 0.05 vs SAH group.
§ p < 0.01 vs sham group.

Fig. 2. Representative sections showing immunohistochemical staining of the basilar artery. A–D: Staining of ox-LDL. Staining was evident throughout the entire layer of the arterial wall in specimens from the SAH group, and the staining was especially intense at the subendothelial layer and the adventitia. In the specimens from the sham group, staining was absent or less evident throughout the entire layer of the arterial wall. In the specimens from the polyphenol groups, staining was absent at the smooth muscle layer, but there was faint staining at the adventitia. E–H: Staining of LOX-1. Staining was extensive throughout the entire layer of the arterial wall in the specimens from the SAH group. In the sham group, the low-dose polyphenol group, and the high-dose polyphenol group specimens, staining was less evident throughout the entire layer of the arterial wall. I–L: Staining of eNOS. Staining was either absent or less evident throughout the entire layer of the arterial wall in the SAH group. In both the sham group and the high-dose polyphenol group, staining was obvious at the endothelium. In the low-dose polyphenol group, staining was faint. Original magnification ×400.
Oxidized-LDL and LOX-1 in cerebral vasospasm after SAH

because arterial luminal narrowing was ameliorated in the accompaniment of decreased expression of ox-LDL and LOX-1 in the polyphenol group, in a dose-dependent manner. Their roles in the pathogenesis of cerebral vasospasm are considered as resulting from the generation of intracellular ROS and causing lipid peroxidation followed by activation of several intracellular signaling transduction pathways associated with smooth muscle contraction and downregulating eNOS at endothelial cells (Fig. 6C), which was confirmed in the SAH group in this study. In any case, this study is the first report to suggest that expression of ox-LDL and LOX-1 at the arterial wall increases after SAH. Details regarding roles of ox-LDL and LOX-1 in the pathogenesis of cerebral vasospasm should be elucidated in the future.

In terms of prevention or treatment of cerebral vasospasm, because free radicals and ROS have been considered main causative factors, the scavenging of free radicals in the subarachnoid space is expected to ameliorate cerebral vasospasm, and several radical scavengers have been tried. However, as of now these are not commercially available. In this study we used apple polyphenol, a natural substance, because it contains abundant procyanidins. The potency of procyanidin as a radical scavenger is considered stronger than ebselen, because quercetin, a polyphenol with the same radical scavenging activity as procyanidin, has been proven to show a radical scavenging activity 10 times higher than ebselen.

Procyanidin is immediately absorbed after ingestion, and the blood concentration increases within 1 hour in a dose-dependent manner. After being absorbed in blood, it is distributed to various tissues and organs, including the brain, within several hours of ingestion, which suggests that procyanidins can pass the blood-brain barrier. The dosage of apple polyphenol administered in the low-dose group in this study was 10 mg/kg, which is the same as the dose per body weight used in the study investigating the usefulness of apple polyphenol in the primary prevention of metabolic syndrome. And the dosage of apple polyphenol administered in the high-dose group in this study is about 5 times higher than in that same study. An experimental study using rats has shown that no harmful
effects are seen at a dose of 2000 mg/kg administered for 90 days, which indicates that the no observed adverse effect level (NOAEL) of apple polyphenol is greater than 2000 mg/kg.44

As to the changes obtained in the polyphenol group, staining of ox-LDL at the smooth muscle layer, including the subendothelial layer, was markedly reduced in the immunohistochemical analysis, which may indicate that the effect of apple polyphenol extended across the entire layer of smooth muscle cells. On the other hand, staining of ox-LDL at the adventitia remained in the polyphenol group. LDL that derives from a subarachnoid clot and is oxidized at the ultra-early stage after SAH, and therefore before the radical scavenging effect of apple polyphenol can be achieved,44 might remain in the form of ox-LDL29 and be immunohistochemically stained at the adventitia.

Decreased expression of ox-LDL may cause the marked reduction of LOX-1 expression seen in the polyphenol group in this study. Additionally, procyanidins have been reported to play a role as an LOX-1 inhibitor by inhibiting ox-LDL from binding to LOX-1,26 which may assist the inactivation of LOX-1. A vicious cycle of ox-LDL, LOX-1, and ROS can therefore be suppressed by the procyanidins contained in apple polyphenol, resulting in the increased expression of eNOS and a significant increase in the lumen of the basilar artery in a dose-dependent manner as compared with the SAH group. Therefore, we believe that apple polyphenol is a candidate for preventive treatment of cerebral vasospasm and that this should be investigated in a clinical study.

We also tried to elucidate how the brain was affected in each group by evaluating apoptosis in the cortex, because early brain injury including apoptosis has been in focus as a factor contributing to poor outcome after SAH.4 It has been reported that early brain injury was ameliorated by suppressing oxidative stress.34 We therefore

![Fig. 5. Representative photomicrographs showing TUNEL (A–D) and H & E (E–H) staining of cerebral cortex sections. There are no TUNEL-positive cells in any of the groups. H & E staining shows no difference among any of the groups. Original magnification ×200.](image)

**Fig. 5.** Representative photomicrographs showing TUNEL (A–D) and H & E (E–H) staining of cerebral cortex sections. There are no TUNEL-positive cells in any of the groups. H & E staining shows no difference among any of the groups. Original magnification ×200.

![Fig. 6. Schematic illustration of the mechanisms and roles of ox-LDL and LOX-1 in the pathogenesis of cerebral vasospasm. A: Initially, LDL in the arterial wall is converted to ox-LDL by the action of ROS derived from the subarachnoid clot. The LDL at the adventitia might originate from subarachnoid blood existing around the major arteries. The source of LDL at the subendothelium might be circulating blood, since the permeability of vessel endothelium is increased after SAH. Ox-LDL triggers upregulation of LOX-1 at the endothelial cells and smooth muscle cells. B: Ox-LDL and LOX-1 are easily combined and increase intracellular ROS, which can, in turn, oxidize LDL. ROS in the endothelial cells increase the permeability of vessel endothelium, facilitating the infiltration of LDL from circulating blood into the arterial wall. C: In addition to the action of free radicals and ROS in the subarachnoid clot, ROS in the smooth muscle cells cause their contraction by activation of associated intracellular signaling transduction pathways, and ROS in the endothelial cells cause changes in those cells, including downregulation of eNOS.](image)
expected that apple polyphenol could ameliorate early brain injury. However, we could not detect any apoptotic cells in the specimens from any of our groups. Experiments on early brain injury have been performed using the endovascular puncture SAH model, which causes a sudden increase in intracranial pressure similar to human SAH. We hypothesize that the cisterna magna puncture rabbit SAH models used in this study lacked the sudden increase in intracranial pressure and failed to cause early brain injury. We plan to carry out another investigation using an endovascular puncture SAH model to evaluate the efficacy of apple polyphenol for the prevention of early brain injury.

Conclusions
This is the first report to reveal that the expression of ox-LDL and LOX-1 at the arterial wall increases after SAH. Administering apple polyphenol inhibited the expression of ox-LDL and its receptor LOX-1, increased the expression of eNOS at the arterial wall, and inhibited narrowing of the basilar artery (reduction of its cross-sectional area) after SAH in a dose-dependent manner. Therefore, it is assumed that ox-LDL and LOX-1 may be implicated in the pathogenesis of cerebral vasospasm, and that procyanidins in apple polyphenol may be a candidate for preventive treatment of cerebral vasospasm by action inhibiting a vicious cycle of ox-LDL, LOX-1, and ROS.

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Disclosure
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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References
42. Shafiee M, Carbonneau MA, Urban N, Descomps B, Leger CL: Grape and grape seed extract capacities at protecting LDL from oxidation generated by Cu2+, AAPH or SIN-1 and at decreasing superoxide THP-1 cell production. A comparison to other extracts or compounds. Free Radic Res 37:573–584, 2003

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