Original Investigation

The Effects of Proanthocyanidin on Vasospasm After Experimental Subarachnoidal Hemorrhage in Rats

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ABSTRACT

AIM: Delayed ischemic neurological deficit (DIND) and cerebral vasospasm (CV) remain the most common and debilitating neurological complications following aneurysmal subarachnoidal hemorrhage (SAH). Many reports demonstrate the importance of proanthocyanidins (PR) on the vascular system, including endothelium-dependent relaxation of blood vessels. These effects of PR on the cerebral vascular system were examined in this study.

MATERIAL and METHODS: Fifty-two adult Sprague-Dawley male rats were used for the experimental double hemorrhage model. They were divided to control, sham, pre- and post-interventional treatment groups. 100 mg/kg PR was administered for the treatment for respect to groups. Basilar artery diameter (BAD) and arterial wall thickness were measured and the apoptosis ratio of the endothelial cells was calculated. Arterial walls were examined electron microscopically (EM).

RESULTS: There were significant differences between the groups except control and pre-SAH (p=0.37) and post-SAH and pre-SAH groups (p=0.15) with respect to BAD. According to arterial wall thickness, apoptosis ratio, and grading, there were significant differences between the groups except control and pre-SAH (p=0.85, p=0.49 and p=0.18 respectively) and SAH and post-SAH (p=0.08, p=0.21 and p=0.24 respectively) groups. EM findings revealed that pro-apoptotic and pro-necrotic degenerated endothelial cells with seldom vacuolization in post-SAH treatment group which were more serious in SAH group.

CONCLUSION: Pre-SAH administration of PR induces better vasodilatation and protection of basilar artery (BA) from vasospasm (VS), which could yield neuroprotective and vasodilatator effects. In addition, PR appears to be involved in relieving oxidative damage, with an antioxidant-antiapoptotic-antinecrotic effect that may contribute to vascular dilation.

KEYWORDS: Subarachnoidal hemorrhage, Vasospasm, Proanthocyanidin, Antioxidant

■ INTRODUCTION

Delayed ischemic neurological deficit (DIND) and cerebral vasospasm (CV) remain the most common and debilitating neurological complications following subarachnoid hemorrhage (SAH) (37). Twenty-five to 30% of patients who survive SAH from a ruptured intracranial aneurysm suffer from ischemic complications in the ensuing weeks, and 10 to 15% of patients with SAH die or experience permanent disability due to their ischemic deficits (18,21).

Although there is some controversy regarding the correlation between cerebral vasospasm in large arteries and cerebral ischemia, vasospasm remains one of the primary causes of DIND (25,35). Proven therapeutic options for the treatment and/or prevention of DIND or CV are few. Suarez et al., summarized current treatment recommendations for vasospasm as consisting of prophylactic nimodipine; hypervolemia with induced hypertension, usually following the onset of vasospasm with neurological deficits; and a potential for balloon

angioplasty or directed application of vasodilating agents (37). Due to the emergence of the more rigorous principles of evidence-based medicine, the need has arisen for a review of the available evidence on which to base the prevention and treatment of vasospasm in patients suffering from aneurysmal SAH.

Proanthocyanidins (PR) are plant condensed tannins with known properties of antimicrobial, antiviral, antitumoral, antioxidant, anti-inflammatory, antiulcer, and anti-diarrheal activity (12). Their regulatory effects on the cardiovascular system have also been demonstrated. These regulatory effects mainly include decreasing expression of VCAM-1, ICAM-1, and E-selectins induced by tumor necrosis factor-α (TNF-α) and limiting platelet aggregation. Proanthocyanidins also have an effect on nitric oxide (NO) and NO synthase (NOS) activity that are believed to have a very important role in the cerebral vasospasm mechanism (12).

The endogenous NOS (eNOS) activity is regulated by mechanisms such as expression and transcription of NOS, subcellular localization (protein-protein interactions and acylation of eNOS), regulation of NOS enzymatic activity by effects on the NOS protein (calcium/calmodulin and reversible palmitoylation), phosphorylation, and S-nitrosylation (14,15). Intracellular calcium is increased by mobilization from the stores and also $Ca2+$ entry via voltage-independent cation channels and this results in activation of eNOS (4,5,13,23).

Phospholipase C (PLC), phospholipase A2 (PLA2), tyrosine kinase, or protein kinase (PKC) activities are also reported to be associated with the increase of $Ca2+$ (1,2).

The aim of this study was to evaluate the PR-induced vasodilator and antioxidant effect in the rat basilar artery after experimental vasospasm.

■ MATERIAL and METHODS

The Animal Care and Use Committee Institution at Baskent University approved the protocol for this study. The rat double hemorrhage model was used for inducing vasospasm (36,39).

Male Sprague-Dawley rats (n=52) each weighing 380 to 420 g were anesthetized by intraperitoneal injection of ketamine (Ketalar, Parke-Davis, Eczacibasi, Istanbul,Turkey) (100 mg/kg) and xylazine (Rompun, Bayer, Istanbul, Turkey) (10 mg/kg). The animals were allowed to breathe spontaneously in the supine position and their body temperature was set to approximately 37°C by using heating pads. The inguinal region was prepared in a standard sterile manner, and the right femoral artery was exposed so that arterial blood could be obtained by cannulation. A suboccipital incision was made and nuchal muscles were divided bilaterally in order to expose the atlantooccipital membrane. A 26 -gauge needle was inserted into the cistern magna via an atlantooccipital membrane puncture and 0.2–0.3 ml of cerebrospinal fluid (CSF) was aspirated. Then 0.2 ml of non-heparinized autologous arterial blood was withdrawn from the femoral artery and injected into the cisterna magna over 2 minutes to simulate SAH. Sham group rats were injected with saline instead of blood.

After injection, the muscles and the skin incision were sutured in layers. The rats were placed in a head-down position for 20 minutes to ensure rostroventral blood distribution around the basal intracranial arteries. The rats remained on the heating pad and were monitored closely until they recovered. After the recovery, 100 mg/kg PR was initiated to the treatment groups.

The second SAH was induced with the same procedure on the second day with the sham group receiving saline again. The rats were randomly divided into four groups, each having 13 animals:

1. For control (n = 13), 0.2 mL of 0.9% NaCl was injected into the subarachnoid space via puncture of the cisterna magna.

2. For the sham group (n = 13), 0.2 mL of autologous arterial blood was injected into the subarachnoid space via puncture of the cisterna magna.

3. For the post-interventional treatment group (n =13), 0.2 mL of autologous arterial blood was injected into the subarachnoid space via puncture of the cisterna magna and 100 mg/kg PR was initiated orally (with gavage feeding tube) after the recovery of the rats from anesthesia and continued for five days at 100 mg/kg/day.

4. For the pre-interventional treatment group (n = 13), 100 mg/kg PR was initiated orally (with gavage feeding tube) 24 hours before the surgical intervention and 0.2 mL of autologous arterial blood was injected into the subarachnoid space via puncture of the cisterna magna and 100 mg/kg PR was administered after the recovery of the rats from anesthesia and was continued for five days at 100 mg/kg/day.

PR Preparation:

We dissolved PR in 2 ml water.

Sacrificing of Animals and Sample Preparation

Five days after the second cisterna magna puncture, 8 rats from surgical groups were sacrificed in deep anesthesia and underwent perfusion-fixation by thoracotomy. The ascending aorta was cannulated, perfusion fixation via intracardiac infusion of 100 mL of 0.03 M phosphate buffer (pH 7.4), pre-warmed to 37 °C, and set up at a height of 100 cm was initiated, followed by 200 mL of 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer.

The brains of the animals were removed carefully after craniectomy and harvested basilar arteries were placed in paraformaldehyde and 1% glutaraldehyde in phosphate buffer solution and stored at +4°C. Paraffin-embedded tissues were cut at a thickness of 0.5 micrometers and stained with Hematoxylin and eosin (HE) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) staining was performed in order to show apoptosis.

Measuring the arterial diameter: Multiple arterial cross-sections were obtained from each animal and after excluding irregular shapes, five specimens were randomly selected. The specimens were studied by a pathologist using ocular micrometer (x20, 0.05 mm, 0.5 mm) in order to measure the luminal cross-sectional areas. An average of the five measured

specimens was calculated for each animal. Group data were shown as mean±standard error.

Staining for apoptosis: Immunohistology using the In Situ Cell Death Detection Kit, POD (Roche, Germany) was used to demonstrate apoptosis in a cross section of basilar arteries. Paraffin embedded samples were deparaffinized and re-hydrated in decreasing concentrations of alcohol. Samples were first treated with proteinase K (20 mg/mL) for 15 minutes at room temperature to increase permeability of the samples. After endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 minutes, 75 µl of equilibration buffer was applied on the sections. The samples were then treated with TUNEL reaction mixture, which catalyzes polymerization of nucleotides to free 39-OH DNA ends in a template independent manner, used to label DNA strand breaks, in a humidified chamber for 1 hour at RT. Sections were then incubated with Converter-POD for 30 min. and then to develop color, 3-Amino-9-ethylcarbazole (AEC) was applied for 12 min. Next, they were counterstained with methyl green and mounted. Between steps, the slides were washed in phosphate-buffered saline (PBS). Sections of the arteries showed staining in the nuclei of the endothelial cells. Apoptotic index was calculated as the number of the immunoreactive nuclei per total number of endothelial cells, expressed as a percentage.

Grading of the apoptosis: Apoptosis in basilar artery endothelial cells was evaluated. The ratio of the cells with positive nuclear staining to all endothelial cells in one section was calculated. Thus, approximately 80 endothelial cells were counted in every artery. The grading was established according to the percentages of the apoptotic cells (Grade 1: 0-25%; Grade 2: 25-50%; Grade 3: 50-75%; Grade 4: 75-100%)

For transmission electron microscopy (TEM), three specimens from the surgical groups were placed in buffered 1% osmium tetroxide, dehydrated in a graded ethanol series and then embedded in Epon 821. They were examined under a JEOL 100C transmission electron microscope (JEOL Inc., Tokyo, Japan). For scanning electron microscopy (SEM), specimens were placed in cacodylate-buffered 2.5% glutaraldehyde for 3 h and post-fixed in 1% osmium tetroxide in the same buffer

for 1 h. They were then dehydrated in a graded alcohol series and amyl acetate and critical-point dried with CO_2, coated with gold in an evaporator and examined under a JEOL scanning electron microscope.

Statistical Analysis

We measured the differences of mean values (arterial diameter, arterial wall thickness and apoptosis grading and ratio) analyzed with one-way variance analysis (One-Way ANOVA) and post hoc Tukey test. A "p" value of less than 0.05 was considered statistically significant. Results were expressed as mean, standard deviation (SD), median and range.

■ RESULTS

Three animals from SAH and one from the post-treatment groups died on the 2nd day of the operation and were excluded from study and replaced with new subjects. The macroscopic examination showed clot covering the brain stem and basal cistern of the subjects. Sections of basilar arteries demonstrated the differences in the diameter and wall-thickness of basilar arteries from each group under light microscopic evaluation (Figure 1A-D).

Luminal Diameters and Thickness: The measurements of luminal diameters and thickness are seen in Figure 3 and Table I. According to the measurements and assuming the luminal diameter there were significant differences between the groups except control and pre-SAH (p=0.37) and post-SAH and pre-SAH groups (p=0.15).

According to the measurements and assuming the arterial wall thickness there were significant differences between the groups except control and pre-SAH (p=0.85) and post-SAH and SAH groups (p=0.08).

According to apoptosis ratio and grading there were significant differences between the groups except control and pre-SAH (p=0.49 and p=0.18) and SAH and post SAH (p=0.21 and p=0.24) groups.

Electron Microscopy Findings: Normal basilar artery lumen was seen in control group (Figure 2A). Electron microscopy revealed vacuolization of the endothelial cells in the tunica

Table I: According to the Measurements and Assuming the Luminal Diameter There were Significant Differences Between the Groups Except the Control-Pre-SAH and Post-SAH-Pre-SAH Groups. According to Arterial Wall Diameter, Apoptosis Ratio and Grading There were Significant Differences Between the Groups Except the Control-Pre-SAH and SAH-Post SAH Groups

Compared groups	Arterial Diameter (p)	Arterial Wall Thickness (p)	Apoptosis Ratio (p)	Apoptosis Grade (p)
SAH-Control	<0.0001	0.001	0.008	0.009
SAH-post-SAH	0.030	0.080	0.213	0.242
SAH-pre-SAH	0.023	0.002	0.036	0.05
Control-post-SAH	0.027	0.034	0.046	0.034
Control-pre-SAH	0.371	0.847	0.494	0.178
post-SAH-pre-SAH	0.154	0.019	0.034	0.070

intima layer with the expression of lamellar shaped myelin figures, loosening and degeneration of internal elastic layer, irregularity and irregular extension of endothelial cells and tight, irregular and flat extensions between the endothelial cells in the SAH group (Figure 2B).

Even pro-apoptotic and pro-necrotic degenerated endothelial cells with seldom vacuolization can be seen in post-SAH treatment group. However normal endothelial cells and internal elastic membrane were seen mostly (Figure 2C).

◼ DISCUSSION

After the investigation of the pathophysiology of cerebral vasospasm in numerous studies, the prevalent ideas about the mechanism are altered calcium homeostasis and increased vascular contractility. Its believed that the first step is the elevation of intracellular calcium levels in the arterial smooth muscle cells, and that hemoglobin (Hb) is responsible for this increased calcium (19,38). The responsibility of ferrous hemoglobin released from blood clots to the vasospasm was shown before, but this effect was temporary (8,22,41).

Actually hemoglobin might show its contractile effect by scavenging nitric oxide, destroying neuronal nitric oxide synthase (NOS) in the adventitia layer and disturbing the function of endothelial NOS (24,28,32). Prolonged decrease of NO can cause prolonged pathological arterial contraction independent from intracellular calcium concentration. Endothelins are also stimulated by hemoglobin resulting in a nitric oxide and endothelin imbalance (16,28). Many clinical and experimental studies were performed about the endothelin receptor antagonist mechanism but they were inconclusive and did not lead to a reduction in the mortality and morbidity of SAH (27).
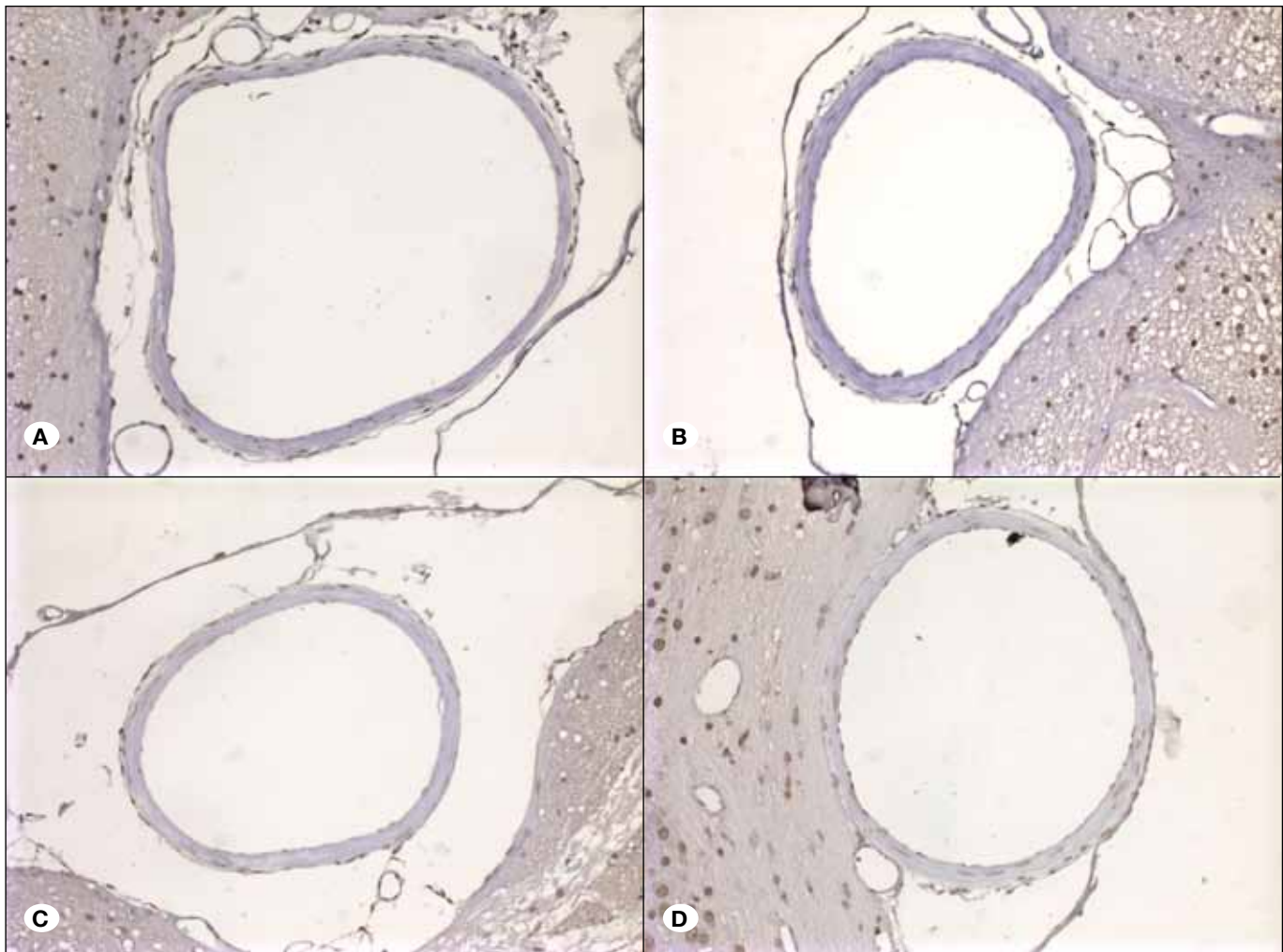

Figure 1: A) The lumens of the basilar arteries in the control group were covered by a simple lining of endothelial cells. The tunica media consisted of well-arranged smooth muscle cells. The tunica adventitia contained loosely arranged connective tissue cells. **B)** There was marked curling and thickening in the elastic lamina and swelling in the endothelial cells of the basilar arteries from the SAH groups. Endothelial cell membranes were cigar-like in shape and there were separations between neighboring elastic laminas. Smooth muscle cells in tunica media demonstrated swelling, thickening, shortening and vacuolization. **C)** In the post-treatment groups, there was mild curling and prominence of the elastic lamina and mild swelling of endothelial cells. **D)** The endothelial cell membrane kept its normal shape. Smooth muscle cells in the media layer demonstrated shortening and thickening and mild vacuolization.

The early phase of the vasospasm after SAH was determined to shos increased intracranial pressure, disruption of the blood–brain barrier (BBB), global cerebral ischemia, inflammation and cortical spreading depression (17,26,29,34). These phases can cause delayed arterial narrowing in determining poor outcome following SAH in the late phase (22). As shown in our study, vasospasm is not only the contraction of the arterial wall. Electron microscopy revealed vacuolization of the endothelial cells in the tunica intima layer with the expression of lamellar shaped myelin figures, loosening and degeneration of internal elastic layer, irregularity and irregular extension of endothelial cells and tight, irregular and flat extensions between the endothelial cells. There were pro-apoptotic or pro-necrotic degenerated endothelial cells in the SAH and treatment groups at different levels. This degeneration can be established in every cerebral artery, which can be surrounded by CSF, which is filled with Hb brake-down products.

PRs, or plant condensed tannins, consist of a group of polyhydroxyl-flavan-3-ol (or flavan-3,4-diol) oligomers and polymers linked by carbon-carbon bonds between flavanol subunits. They are the second most abundant natural phenolic compound class after lignins (30) . They are widely found in fruits, wine, cocoa, nuts, berries and beans and therefore consumed extensively by humans (30).

Vegetable tannins are also used in many medicinal plants used for a range of diseases. In a recent review by De Bruyne et al. several properties such as antimicrobial, antiviral, antitumoral,
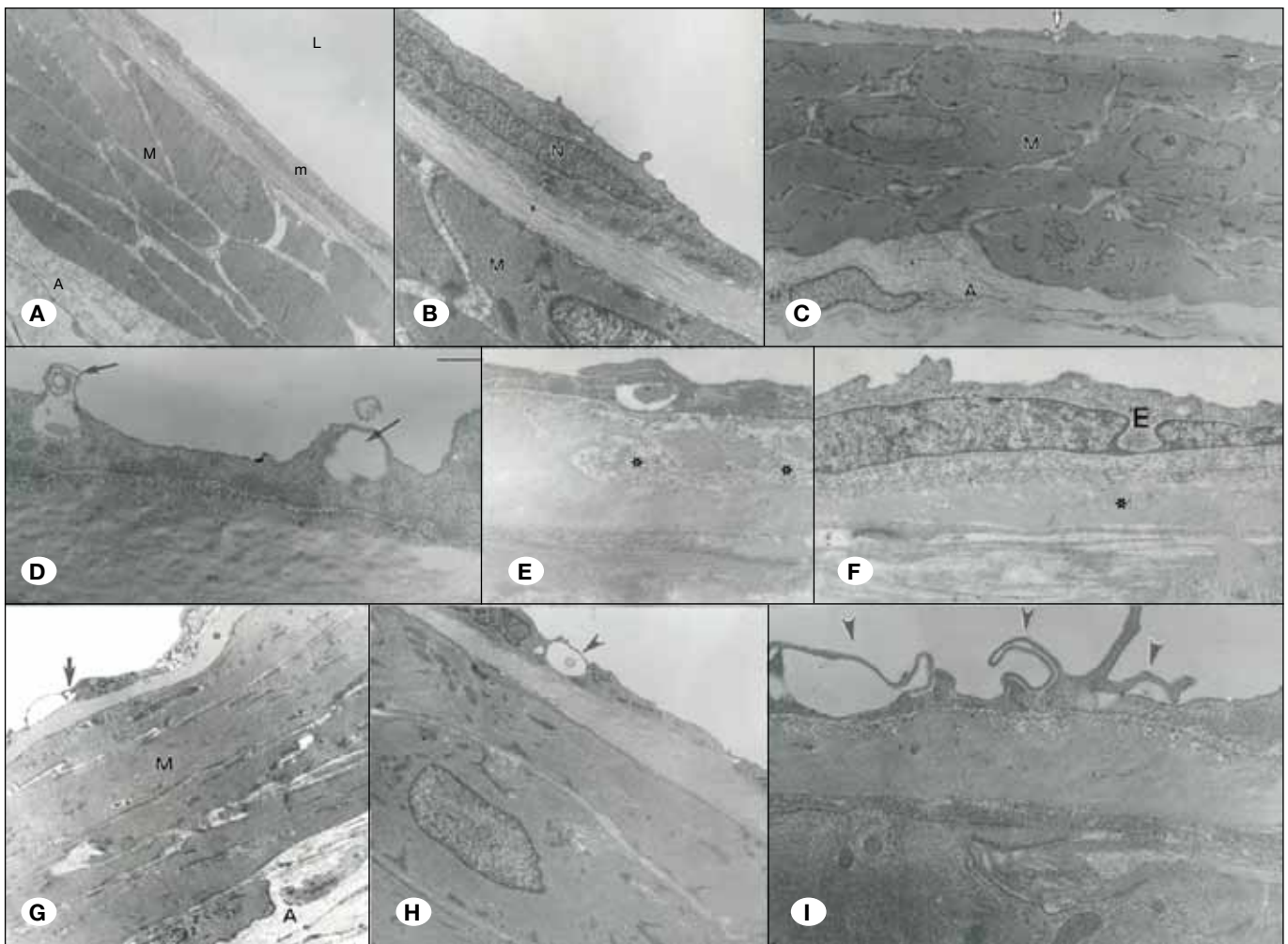

Figure 2: Group 1 A) Group 1: Basilar Artery Lumen (L), endothelial cells in tunica intima layer (E) and internal elastic membrane (asterisks), the shape of the smooth muscle cells in tunica media layer (M), nucleus and indigents of the cytoplasm and adventitia (A) seem to be normal with connective tissue. **B)** Group 1: In the higher magnification, endothelial cells (E) and internal elastic membrane (asterisks), smooth muscle cells in tunica media layer (M) were seen in the Basilar Artery. **Group 3 C**) Smooth muscle cells in the tunica media layer (M) and adventitia layer seem to be normal but the vacuolization (arrow) in endothelial cells in the tunica intima layer was seen. **D)** Huge vacuolization with lamellar myelin figures (arrow) in endothelial cells, **E)** Loosening type degenerative changes in the internal elastic membrane layer (asterisks) **F)** Irregular extensions in endothelial cells (arrows); irregularity and irregular extension of endothelial cells and tight, irregular and flat extensions between the endothelial cells. **Group 2 G)** Highly degenerated endothelial cells; pro-apoptotic and pro-necrotic endothelial cells (arrow). **H)** Endothelial cells with huge vacuolization (arrow) **I)** Endothelial cells with normal formation and (E) and internal elastic membrane (asterisks).
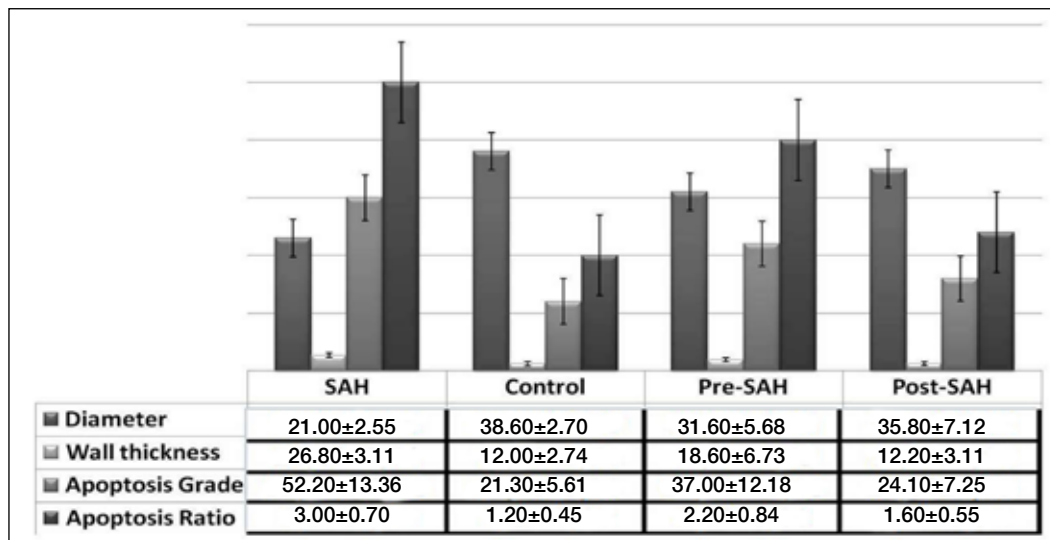
	SAH	Control	Pre-SAH	Post-SAH
▪ Diameter	21.00±2.55	38.60±2.70	31.60±5.68	35.80±7.12
▪ Wall thickness	26.80±3.11	12.00±2.74	18.60±6.73	12.20±3.11
▪ Apoptosis Grade	52.20±13.36	21.30±5.61	37.00±12.18	24.10±7.25
▪ Apoptosis Ratio	3.00±0.70	1.20±0.45	2.20±0.84	1.60±0.55

Figure 3: The measurements of luminal diameters, arterial wall thickness, apoptosis ratio and grading.

antioxidant, anti-inflammatory, anti-ulcer, and anti-diarrheal activities were described (12).

Taken together, these studies demonstrate that grape seed proanthocyanidin extract (GSPE) is a bio-available and potent free radical scavenger, and exhibits novel cardioprotective properties. The novel mechanistic pathways of cardioprotection exerted by GSPE include: (i) potent hydroxyl and other free radical scavenging abilities; (ii) anti-apoptotic, anti-necrotic, and anti-endonucleolytic potentials; (iii) modulatory effect on apoptotic regulatory bcl-XL, p53, and c-myc genes; (iv) cytochrome P450 2E1 inhibitory activity; (v) inhibitory effects on adhesion molecules; (vi) modulatory effects on proapoptotic and cardioregulatory genes c-JUN, JNK-1, and CD36 (3). Electron microscopy of the treatment group and TUNEL staining of all groups with significant different rates of apoptosis of the arterial wall showed the antioxidant-antiapoptotic-antinecrotic effect of PR.

NO can exert a variety of biological activities in various physiological and pathological conditions. As a free radical, NO has been shown to play a double-edged role in the regulation of apoptosis. It can be either pro-apoptotic or anti-apoptotic, depending on the type of cell, the location, the timing and the amount produced (40). Recent studies have shown that GSPE increases NO by activating the inducible NOS (iNOS) (31). The vasodilator effect of PR was shown in pre- and post-treatment groups.

PRs have been shown to have effects on the cardiovascular system. They upregulate the activity and expression of super-oxide dismutase, and have antioxidant activity and ability to augment the antioxidant potential of plasma in rats (6,9,20). They augment the biological activity of nitric oxide (NO) and NO synthase expression (33).

We demonstrated the vasodilator effect of PR in the pre-treatment and post-treatment group with significant difference of arterial diameter with respect to SAH group. There was no significant difference with respect to the control group. An antioxidant effect was shown with electron-microscopical findings of the structural investigation of the arterial walls of the groups. There were milder findings of pathological changes due to vasospasm in the treatment group with respect to the vasospasm group (Figures 2D-I).

Endothelial vasodilation has been extensively studied and found to be controlled mainly by NO, prostacyclin, and endothelium derived hyperpolarizing factor (7).

DalBó et al., as a result of their study aiming to investigate the relaxing effects of a proanthocyanidin in the rat mesenteric arterial bed, found that this effect involves mainly the NO–cGMP pathway and hyperpolarization induced by KCa2+ activation (10,11).

Since PRs are found in the normal human diet, evaluation of their clinical efficacy for potential application for the prevention and treatment of cardiovascular diseases is important as this plant enhances endothelial function by formation of vasoprotective endothelial factors. In our study, we showed the protective effect of PR in the basilar artery by a lower apoptosis level of the basilar artery in the pre-treatment group.

■ **CONCLUSION**

Pre-SAH administration of PR induces better vasodilatation and protection of BA from VS, which could yield neuroprotective and vasodilatator effects. In addition, PR appears to be involved in relieving oxidative damage, with an antioxidant-antiapoptotic-antinecrotic effect that may contribute to vascular dilation. Further studies are needed to confirm these results and to elucidate the possible biochemical mechanisms underlying the action of PR in preventing CV.

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