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What is the restorative effect of VEGF inhibitor bevacuzimab against subarachnoid hemorrhage in an experimental model?

Adnan Yalçın DEMİRCİ[1], Yahya GÜVENÇ[2], Ersin ÖZEREN[3], Pınar BAYRAM[4], Deniz BİLLUR[5], Sevim AYDIN[5], Çetin AKYOL[6], Hakan SEÇKİN[7], Kazım YİĞİTKANLI[7,*]

[1]Department of Neurosurgery, Yüksek İhtisas Education and Training Hospital, Bursa, Turkey
[2]Department of Neurosurgery, Faculty of Medicine, Marmara University, İstanbul, Turkey
[3]Department of Neurosurgery, Faculty of Medicine, Aksaray University, Aksaray, Turkey
[4]Department of Histology and Embryology, Faculty of Medicine, Kafkas University, Kars, Turkey.
[5]Department of Histology and Embryology, Faculty of Medicine, Ankara University, Ankara, Turkey
[6]Department of Neurosurgery, Yıldırım Beyazıt Training and Research Hospital, Ankara, Turkey
[7]Neurosurgery Clinic, Medicana Bursa Hospital, Bursa, Turkey

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Background/aim: This study investigated the effect of vascular endothelial growth factor (VEGF) inhibitor bevacuzimab (BVZ) on the rabbit basilar artery using an experimental subarachnoid hemorrhage (SAH) model.

Materials and methods: Eighteen adult male New-Zealand white rabbits were randomly divided into three groups: a control group (n = 6), SAH group (n = 6), and SAH+BVZ group (n = 6). Experimental SAH was created by injecting autologous arterial blood into the cisterna magna. In the treatment group, the subjects were administered a daily dose of 10 mg/kg, intravenous BVZ for 2 days after the SAH. Basilar artery diameters were measured with magnetic resonance angiography (MRA) 72 h after the SAH in all groups. After 72 h, whole brains, including the upper cervical region, were obtained from all the animals after perfusion and fixation of the animal. The wall thickness, luminal area, and the apoptosis at the basilar arteries were evaluated in all groups.

Results: BVZ significantly prevented SAH-induced vasospasm confirmed in vivo with MRA imaging with additional suppression of apoptosis on basilar artery wall.

Conclusion: VEGF inhibition with BVZ has shown to have a vasospasm and apoptosis attenuating effect on basilar artery in a SAH model.

Key words: Apoptosis, bevacizumab, subarachnoid hemorrhage, vasoconstriction, vascular endothelial growth factors

1. Introduction

Cerebral vasospasm is one of the most important causes of mortality and morbidity in subarachnoid hemorrhage. Vasospasm seems to be a multifactorial and complicated process without any clear etiology or effective treatment [1,2]. The endoplasmic reticulum stress-mediated apoptosis pathway is considered to play a vital role in mediating stroke and other cerebrovascular diseases like subarachnoid hemorrhage (SAH) [3].

In clinical settings and experimental SAH models, vascular endothelial growth factors (VEGF) were reported to increase in acute phase of aneurysmal SAH, and were suggested to cause cerebral vasospasm and proliferative angiopathy [4–6]. Therefore, we hypothesized that anti-VEGF therapy may have a restorative effect after SAH. Bevacizumab (BVZ) is a humanized monoclonal antibody with a long half-life (22 days) that inhibits the VEGF. It has been used to treat certain malignancies including breast, lung, renal, and colorectal carcinomas [7]. Available data on the relationship between anti-VEGF therapy and SAH is limited and not well described. VEGF has been shown to stimulate the production of nitric oxide by endothelial cells in arteries and arterioles, resulting in vasodilation [8]. Thus, there is a concern for anti-VEGF therapies causing angiogenic blockage increasing the risk of vasospasm [9,10]. Indeed, retinal arterial vasoconstriction and systemic hypertension have been identified as complications of anti-VEGF therapy [11].

Our study aimed to investigate the effect of VEGF antagonism on cerebral vasospasm and related apoptotic cell death after experimental SAH injury.

* Correspondence: kazimyigitkanli@gmail.com

2. Materials and methods

This study was done under the control of animal experiments local ethic committee at Ankara Training and Research Hospital, Animal Experiments Research Laboratory, Ankara, Turkey between 09/10/2013 and 14/10/2013 (Decision number: 232). The animals underwent the magnetic resonance angiography (MRA) investigations at Dışkapı Y.B. Training and Research Hospital, Ankara, Turkey. The microscopic basilar artery diameter measurements and histopathologic investigations were performed at the Ankara University Medical Faculty's Histology–Embryology Department, Ankara, Turkey (D.B, S.A).

A total of 18 New-Zealand white rabbits (weight range: 2.5–3.0 kg) were used in this study. All subjects were kept under stable and standard environmental conditions during the experiment and received standard animal feed as well as free access to water during the experiment.

2.1. Groups

Three groups of animals were created, each of which consisted of six animals. Control group (Group 1): Animals were given general anesthesia without any surgical intervention. SAH group (Group 2): SAH was created and the group underwent intravascular administration of saline for a total of 3 days starting 2 h after the creation of SAH. Treatment group (Group 3, SAH+BVZ group): 120 min after SAH, 10 mg/kg BVZ (Avastin(R), Genentech, Inc. South San Francisco, CA, USA) was given through intravascular injection. Additional two doses were given 24 and 48 h after SAH.

2.2. SAH model

The rabbits were administered 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine (Alfazyne(R) 2% vial, Ege-Vet, Turkey) intramuscular doses before the surgical procedure. General anesthesia was ensured, and the rabbits were left to their spontaneous respiration. No ventilator support was used during the experiment.

In the subarachnoid hemorrhage model, the occiput and posterior neck of the rabbits were shaved according to aseptic rules. Cerebrospinal fluid (1 mL) was drained from subarachnoid space via cisternal puncture. Auricular arteries of animals in groups 2 and 3 were cannulated via 24-gauge catheter while 2 mlL of blood was drained. Drained blood was slowly injected (after 2 min) to cisterna magna of rabbits in group 2 and group 3. After the blood injection, rabbits were positioned downward for 15 min for the distribution of blood to basal cisterns.

Animals were observed at 23–25 °C. On the 3rd day of the experiment, after performing MRA, the animals were given general anesthesia. Under anesthesia, abdominal and thoracic cavities of the animals were opened. Descending aorta was clamped and the heart was cannulated to aorta via ventricular puncture. Perfusion of the animals was performed via 0.9% NaCl, followed by 4% formaldehyde. The entire brains, including the upper cervical regions, were kept in 10% formaldehyde at 4 °C.

2.3. MR angiography

All subjects were administered 10 mg/kg ketamine hydrochloride (Ketalar vial(R), Pfizer, USA) intramuscularly for sedation on the third day of the study. They then underwent cerebral 1.5-T MRA (Philips Healthcare, Eindhoven, the Netherlands). All MRA images were recorded and measured digitally. Measurements were performed to pass through each basilar artery in five separate segments in the vertical plane, the images were then evaluated statistically.

2.4. Histological study

2.4.1. Tissue preparation

The brain and brainstem were removed and fixed in 10% buffered formalin. For assessment of vasospasm, the entire basilar artery of each animal was collected and sectioned at 5 segments, each being 2 mm in length. The basilar artery samples were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut to 5-μm thicknesses using a microtome (Leica RM 2125RT, Leica, Wetzlar, Germany) and were stained with hematoxylin and eosin (H-E). Slides were examined and photographed using Axio Scope-A1 (Carl Zeiss, Germany) microscope at 100× magnification.

2.4.2. Histomorphometric analysis of the basilar artery

The wall thickness and luminal area of the predetermined five segments of basilar arteries were measured using Axiovision software program (AxioVision, Oberkochen, Germany). The wall thickness was measured at four quadrants of each segment of the basilar artery between endothelium of intima and external border of tunica media. Luminal area was calculated from luminal borders of each segment. The results were recorded and evaluated statistically.

2.4.3. Assessment of apoptotic cells

Under light microscopic examination, the apoptotic cell percentage was calculated by comparing TUNEL-positive stained cells with the complete cell count, including the entire circumferential vessel wall. The examination was done by histologists who were blinded to the study.

For TUNEL staining, immunohistochemical detection of cells undergoing DNA fragmentation was performed using a terminal deoxynucleotidyl transferase (TdT) labeling (TUNEL) method with a commercial in situ apoptosis detection kit (ApopTag Peroxidase in Situ Apoptosis Detection Kit, Millipore, Darmstadt, Germany, S7100). The 5-μm-thick sections were stained according to the manufacturer's protocol for the Peroxidase in Situ Apoptosis Detection Kit. Diaminobenzidine (DAB) was used as a chromogen, and counterstaining was performed using methyl green.

2.5. Statistical analysis

We used means with standard error for continuous variables, respectively plus or minus standard error. A normality test was done prior to analysis. If the normality test revealed that data were normally distributed, statistical differences between the groups were compared by one-way analysis of variance (ANOVA). Then post-Tukey multiple comparison tests were performed if a significant difference had been determined. If the normality test revealed that the data were not normally distributed, the Kruskal–Wallis test was performed. Post hoc analysis was performed between the groups if the data was statistically significant. A probability (p) value of <0.05 was considered statistically significant. Statistical analysis was performed using Prism 9 for Mac (Graphpad Prism, CA, US).

3. Results

All animals survived the 3 days after SAH, and MRA and histopathological assessments were performed for each of the animals.

3.1. MR angiography

The mean artery diameters of the basilar arteries of the MRA was different for the three groups ($p < 0.0001$, the Kruskal–Wallis test). The mean MRA basilar artery diameter was found to be 0.104 ± 0.003 mm in the control group, 0.071 ± 0.011 mm in the SAH group, and 0.089 ± 0.003 mm in the SAH+BVZ group. In the control group, the mean basilar artery diameter was found to be higher than the SAH group ($p < 0.001$). The mean diameter of the artery was higher in the SAH+BVZ group compared to SAH group ($p < 0.05$) (Figure 1, Table).

3.2. Histologic assessment

After removing the brain and the brainstem, widespread SAH was observed macroscopically in the ventral surface of the brain in all SAH and SAH+BVZ groups. A histomorphology assessment was performed on samples with H-E and TUNEL staining. According to H-E staining of the control group, three layers of the basilar artery wall, tunica intima, tunica media, and tunica adventitia, were observed at normal appearance (Figure 2Aa). In the SAH group, narrowed basilar artery lumen, shrunken endothelial cells, corrugated internal elastic membrane, thickened vessel wall, and contracted smooth muscle cells were seen (Figure 2Ab). In the SAH+BVZ group, the basilar artery lumen was larger and the vessel wall was thinner than the SAH group. The internal elastic membrane was smooth, endothelial cells were normally shaped, and smooth muscle cells were elongated (Figure 2Ac).

3.3. Analysis of intergroup wall thickness and luminal area

Evaluation of the basilar artery luminal area showed a significant difference between the three groups ($p < 0.05$, ANOVA). The mean vessel lumen area was found to be 164,624 ± 22,006 μm^2 in the control group, 80,654 ± 17,650 μm^2 in the SAH group, and 125,808 ± 23,916 μm^2 in the SAH+BZV group (Table, Figure 2B). The SAH group showed statistically significant decreased luminal

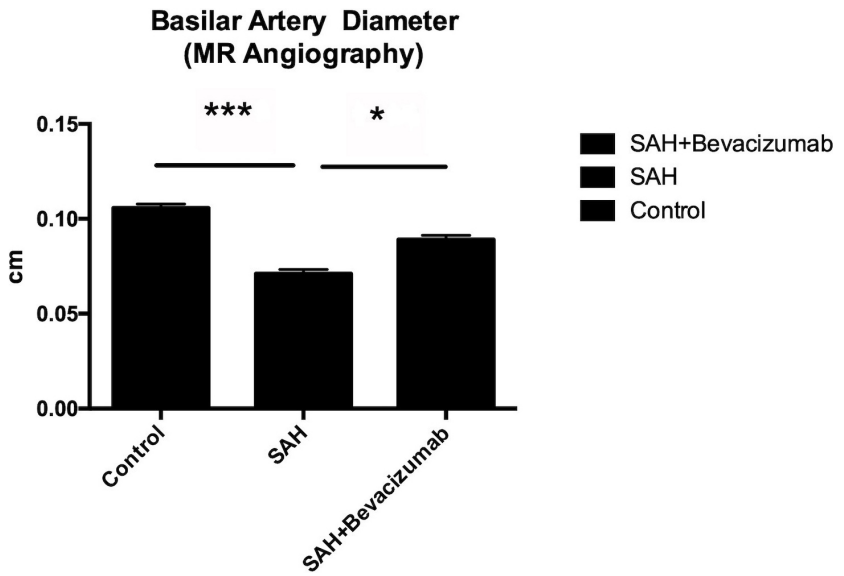

Figure 1. Basilar artery diameter measurements according to in vivo MR angiography compared between groups. There were significant differences between the SAH group and the control group, and also the SAH+BVZ group and the control group (*$p < 0.05$, ***$p < 0.001$). Data is presented as mean ± SE.

Table. The results of measurements.

	Control	SAH	SAH+BVZ	p-value
BA diameter on MRA, (mm) [mean ± SE]	0.104 ± 0.003	0.071 ± 0.048	0.089 ± 0.003	<0.0001‡
Wall thickness (μm), [mean ± SE]	16.69 ± 1.55	31.83 ± 2.75	21.51 ± 1.39	<0.001†
Luminal area measurement (μm²), [mean ± SE]	164624 ± 22006	80654 ± 17650	125808 ± 23916	<0.05†
Apoptotic cell percentage (%), [mean ± SE]	2.67 ± 0.49	83.17 ± 1.96	68.7 ± 2.20	<0.0001‡

BA, basilar artery; BVZ, bevacuzimab; MRA, magnetic resonance angiography; SAH, subarachnoid hemorrhage; SE, standard error. Statistical differences between the groups were compared by ANOVA or the Kruskal–Wallis test according to normality test results († one-way ANOVA test, ‡ the Kruskal–Wallis test).

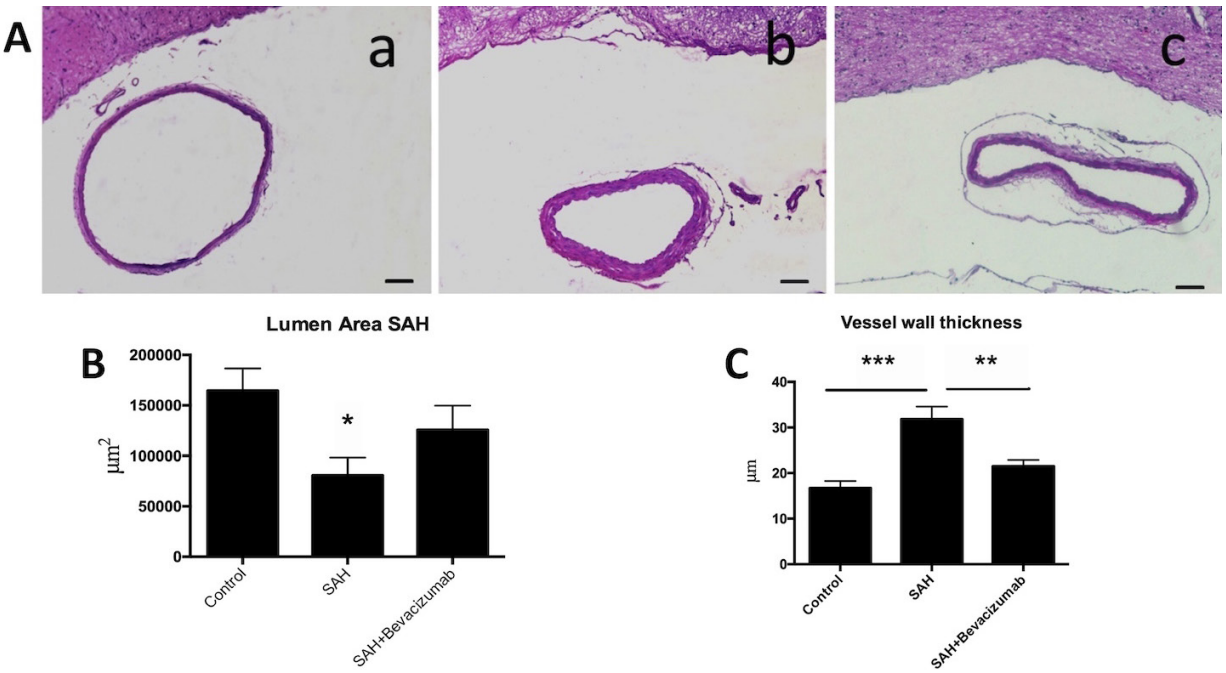

Figure 2. Basilar artery representative images, lumen area and wall thickness results 72 h after subarachnoid hemorrhage. A) HE staining light microscopic images show basilar artery cross sections on the surface of pons; a) Control group, b) SAH group, c) SAH+BVZ group. Scale bar 100 μm. B) Basilar artery lumen areas were compared between groups. There were significant differences at the SAH group compared to the control and SAH+BVZ groups (*$p < 0.05$). C) Basilar artery wall thicknesses were compared between groups. There were significant differences between the SAH group and the control group, and also the SAH+BVZ group and the control group (**$p < 0.01$, ***$p < 0.001$). Measurements are expressed as mean ± SE.

area compared to the control group ($p < 0.05$, Figure 2B). There was even a trend of increase in the luminal area of SAH+BVZ group compared to the SAH group, the difference was not statistically significant ($p > 0.05$). Supporting this trend, there was no statistically meaningful difference between the luminal areas of the SAH+BVZ and the control group ($p > 0.05$).

There was a statistically meaningful difference between wall thicknesses of the groups ($p < 0.001$, ANOVA). The mean vessel wall thickness was found to be 16.69 ± 1.55 μm in the control group, 31.83 ± 2.75 μm in the SAH group, and 21.51 ± 1.39 μm in the SAH+BZV group (Table 1). The SAH group showed statistically significant decrease in vessel wall thickness compared to the control group ($p < 0.001$). The wall thickness was increased in the SAH+BVZ group compared to the SAH group ($p < 0.01$, Figure 2C, Table 1).

3.4. Analysis of intergroup apoptosis measurement difference

The apoptotic cell death in cerebral vessels was determined by TUNEL staining, and the TUNEL (+) endothelium was localized on the inner surface of the corrugated internal elastic membrane (Figure 3A). The means of the apoptotic cell percentage were different from each other in three

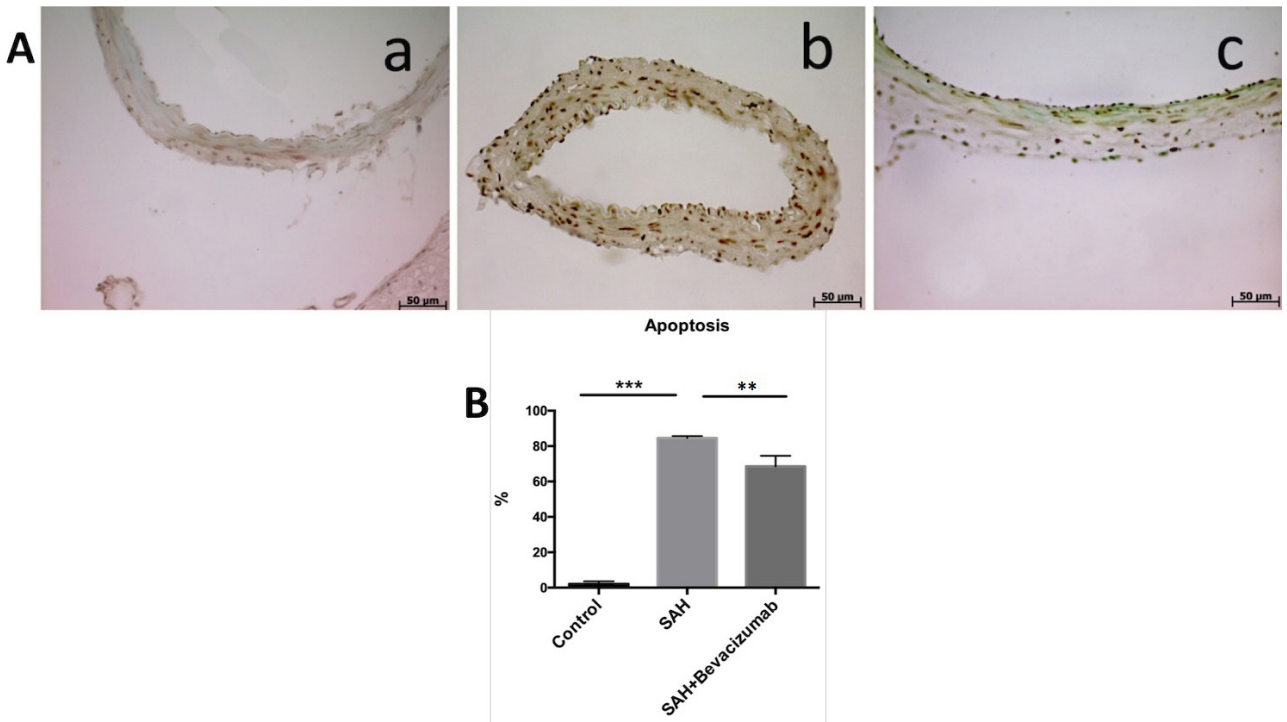

Figure 3. Basilar artery TUNEL staining results 72 h after subarachnoid hemorrhage (SAH). A) The figure shows representative TUNEL staining images for basilar arteries at 72 h after subarachnoid hemorrhage. TUNEL positive apoptotic endothelial cell nucleus (stained with diaminobenzidine [DAB] brown color). Normal endothelial cell nucleus (stained with methyl green); a) Control group, b) SAH group, c) SAH+BVZ group. B) TUNEL positive cell staining results were compared between groups. There were significant differences between the SAH group and the control group, and also the SAH+BVZ group and the control group (**$p < 0.01$, ***$p < 0.001$). Measurements are expressed as mean ± SE.

groups ($p < 0.0001$, the Kruskal–Wallis test). The apoptotic cell percentage was found to be 1.83 ± 0.05 in the control group, 83.16 ± 1.25 in the SAH group, and 62.50 ± 3.80 in the SAH+BZV group (Table 1). In the control group, the mean of the apoptotic cell percentage was significantly lower compared to the SAH group ($p < 0.001$). The means of the apoptotic cell percentage was significantly lower in the SAH+BZV group compared to the SAH group ($p < 0.01$) (Figure 3B).

4. Discussion

In this study, we demonstrated that rabbits that were treated with BVZ after SAH had significantly less vasospasm compared to those that were not treated with BVZ. Additionally, we have also shown that the VEGF pathway might be implicated in the development of cerebral vasospasm with apoptosis pathway on cerebral arteries after SAH.

VEGF were reported to increase in an acute phase of aneurysmal SAH and suggested to cause cerebral vasospasm and proliferative angiopathy in experimental SAH [4–6]. VEGF receptor2 (VEGFR-2), which is a major receptor of VEGF, was reported to be activated after experimental SAH, and VEGFR2 blockage suppresses post-SAH blood-brain barrier (BBB) damage [12]. VEGFR involvement in the pathophysiology of SAH in cerebral arteries was also reported [13]. To the best of our knowledge, there is no previous study evaluating the impact of anti-VEGF treatment effects after SAH.

VEGF has a significant role in vascular permeability and angiogenesis during embryonic vasculogenesis and in physiological and pathological angiogenesis in nonneural vessels. The effects are mediated by VEGFR-2, which is present on endothelial cells [9]. It has also been demonstrated that VEGF enables enhanced BBB permeability in the normal mice brain and inflammatory disease of the mice brain [9]. The cerebral ischemia in animals is usually studied on the model of middle cerebral artery occlusion. These studies revealed an increase of VEGF expression in ischemic areas of the brain [3]. Hypoxia strongly induces VEGF expression in vivo and in vitro models. Animal studies revealed an increase of VEGF expression as early as 3 h after induction of hypoxia, with peak intensity after 48 h [3]. Lei et al. [12] demonstrated that experimental SAH upregulated VEGF expression in the cerebral cortex, causing BBB disruption. Anti-VEGF treatment was found to be protective against post-SAH early brain injury [12]. However, no studies

have investigated the effects of direct blockage of VEGF on cerebral arteries after SAH.

Bevacizumab is an agent used for cancer treatment as a VEGF antagonist. It is a VEGF angiogenesis inhibitor, produced by recombinant DNA technology, and a monoclonal antibody against human VEGF-A. Its molecular weight is 149 kDa. Instead of directly targeting tumor cells, it targets vessels which carry oxygen and nutrition to tumor cells [7]. BVZ binds to VEGF and prevents the interaction of VEGF and its endothelial surface receptor. Thus, BVZ inhibits angiogenesis by clearing circulating VEGF effect [14].

Despite initial concerns of life-threatening hemorrhages with anti-VEGF use in patients with brain tumors, a review of 10,598 cancer patients in 57 clinical trials of anti-VEGF therapy, including bevacizumab, showed that the rate of intracranial hemorrhage, even in patients with high-grade glioma and brain metastases, was negligible (<1%) [15]. Additionally, a subsequent review at Memorial Sloan Kettering Cancer Center showed an IPH frequency of 3.7% in cancer patients receiving bevacizumab, which was identical to a 3.6% frequency detected in comparable patients not treated with bevacizumab [16]. A more recent study by the German Glioma Network similarly demonstrated no significant difference in the rate of intracranial hemorrhages with and without BVZ therapy ($p = 0.571$) [17]. Another study suggested that intracranial hemorrhage in high-grade glioma was due to tumor progression, rather than anti-VEGF therapy [18]. On top of all these reports, Lin X et al. [19] reported preliminary evidence that even rechallenging with bevacizumab therapy may be safe. All of these studies support the view that the intracerebral hemorrhage was unlikely to be due to bevacizumab therapy.

There is also another concern for angiogenic blockage increasing the risk of vasospasm [9,10,20]. Anti-VEGF therapies in general are thought to lead to vasospasm in tissues throughout the body via dysregulation of the renin–angiotensin–aldosterone system [21]. VEGF has been shown to stimulate the production of nitric oxide by endothelial cells in arteries and arterioles, resulting in vasodilation [10]. The reduction of the VEGF activity may potentially increase the risk of vasospasm. Indeed, retinal arterial vasoconstriction and systemic hypertension have been identified as complications of anti-VEGF therapy [11]. However, there was a unique case of a reported glioblastoma multiforme patient who demonstrated no clear evidence of vasospasm after SAH in the setting of BVZ treatment. In that case, a delay of 40 days post-SAH was well tolerated without clinical or radiographic sequelae, including vasospasm or further hemorrhage [22]. Our results show the safety of BVZ in an in vivo model of SAH. Additionally, BVZ had a vasodilatory effect on the basilar artery which was confirmed by histopathological and in vivo MRA results.

In our in vivo experiment, the generally accepted SAH model was chosen for this research. Administration of anti-VEGF antibodies reduced the vasospasm on basilar arteries. Pathological staining results also confirmed the antiapoptotic effect of BVZ on basilar arteries of SAH-induced rabbits. Apoptosis is one of the major cell death patterns in ischemic penumbra (ischemic but still viable cerebral tissue) and SAH, particularly induced by the endoplasmic reticulum stress pathway. In our findings, apoptosis of basilar artery sections were identified by TUNEL staining, showing a significantly reduced number of TUNEL-positive cells in the BVZ treatment group than in the SAH model group. These results indicated that the positive effects of anti-VEGF may be partially related to their antiapoptotic effects at any rate. For this series of phenomena, we believe that BVZ neutralizes the high level of endogenous VEGF, which has a vasospastic and apoptotic role at the time of SAH.

In conclusion, our study demonstrated that the application of BVZ has a vasospasm-attenuating effect. Inhibiting endogenous VEGF expression may decrease cerebral vessel damage in vivo via suppressing apoptosis. Vasospasm-reducing effect of VEGF antagonism may be highly valuable for the literature due to concerns of risks relating to the vasospastic effect after anti-VEGF treatment.

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Ethical approval
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. (Ankara Training and Research Hospital (08/10/2013-Decision number: 232): All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest
The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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