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

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 hours after the SAH in all groups. After 72 hours the animals’ 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.

Keywords: 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 haemorrhage. Vasospasm seems to be a multifactorial and complicated process without any clear etiology or effective treatment [1,2]. The endoplasmic reticulum (ER) 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]. So 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]. So 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.

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 (weights 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 them 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 hours after the creation of SAH. Treatment group (Group 3, SAH+BVZ group): 120 minutes 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 hours 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.

Subarachnoid haemorrhage 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 group 2 and 3 were cannulated via 24 gauge catheter while 2ml blood was drained. Drained blood was slowly injected (after two minutes) to cisterna magna of rabbits in group 2 and group 3. After the blood injection, rabbits were positioned downward for 15 minutes for the distribution of blood to basal cisterns.

Animals were observed at 23-25 C°. On the third 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 were performed via 0.9% NaCl, followed by 4% formaldehyde. The entire brain including the upper cervical region, 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, 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 image were then evaluated statistically.

2.4. Histological study:

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 x100 magnification.

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 segments. Results was recorded and evaluated statistically.

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.

**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 Kruskal-Wallis test was performed. Post-hoc analysis was performed between the groups if the data was statistically significant. A probability value of p < 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 three 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, 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 2A_a). 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 2A_b). 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 2A_c).

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 µm² in the control group, 80.654±17.650 µm² in the SAH group and, 125.808±23.916 µm² in the SAH+BZV group (Table, Figure 2B). SAH group showed statistically significant decrease luminal area compared to the control group (p<0.05, Figure 2B). Even there was a trend of increase in luminal area of SAH+BZV group compared to SAH group, the difference was not statistically significant (p>0.05). Supporting this trend, there was no statistically meaningful difference between the luminal area of SAH+BZV and the control group (p>0.05).

There was a statistically meaningful difference between wall thickness 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). SAH group showed statistically significant decrease in vessel wall thickness compared to the control group (p<0.001). The wall thickness was increased at SAH+BZV group compared to 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 groups (p<0.0001, 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 SAH group (p<0.001). The means of the apoptotic cell percentage was significantly lower in SAH+BZV group compared to SAH group (p<0.01) (Figure 3B).
In this study, we demonstrated that rabbits who were treated with BVZ after SAH had significantly less vasospasm compared to those who 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 our best 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 non-neural 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 are 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 vitro models. Animal studies revealed an increase of VEGF expression as early as 3 hours after induction of hypoxia, with a peak intensity after 48 hours [3]. Liu 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 effects of the 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, Bevacizumab is a monoclonal antibody against human VEGF-A. Its molecular weight is 149 kDa. Instead of directly targeting tumor cells, Bevacizumab 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. So 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 = .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]. But there was a unique case of a reported glioblastoma multiforme patient 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 anti-apoptotic 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 anti-apoptotic 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.

References
3. Feng SQ, Zong SY, Liu JX, Chen Y, Xu R et al. VEGF antagonism attenuates cerebral ischemia/reperfusion-induced injury via inhibiting endoplasmic reticulum


Table: The results of measurements

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<tr>
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<th>Control</th>
<th>SAH</th>
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<tr>
<td>BA diameter on MRA, (mm) [mean±SE]</td>
<td>0.104±0.003</td>
<td>0.071±0.048</td>
<td>0.089±0.003</td>
<td>&lt;0.0001†</td>
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<tr>
<td>Wall thickness (μm), [mean±SE]</td>
<td>16.69±1.55</td>
<td>31.83±2.75</td>
<td>21.51±1.39</td>
<td>&lt;0.001†</td>
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<td>Luminal area measurement (μm²), [mean±SE]</td>
<td>164624±22006</td>
<td>80654±17650</td>
<td>125808±23916</td>
<td>&lt;0.05†</td>
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<tr>
<td>Apoptotic cell percentage (%) , [mean±SE]</td>
<td>2.67±0.49</td>
<td>83.17±1.96</td>
<td>68.7±2.20</td>
<td>&lt;0.0001‡</td>
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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 Kruskal Wallis test according to normality test results († One-Way ANOVA test, ‡ Kruskal Wallis test).
Figure 1. Measurements expressed as mean-standard error of basilar artery diameters measured in-vivo with MRA. *P < 0.05, ***P < 0.001

Figure 2
Figure 2. Effects of BVZ on cerebral vasospasm at 72 hours after subarachnoid hemorrhage (SAH). (A). Panel shows representative pictures of basilar artery cross sections with hematoxylin-eosin (HE) staining (a) Control group, (b) SAH group (c) SAH + BVZ group. HE staining light microscopic images show basilar artery cross sections on the surface of pons. The wall thickness was measured at four points of each segment of the basilar artery between the endothelium of the intima and the external border of the tunica media. Increasing arterial wall thickness and narrowing luminal area are seen prominently in the SAH group (b) which was resolved by BVZ treatment (c) HE. Scale bar 100 μm. Degree of vasospasm assessed by lumen area (B) and wall thickness (C). Measurements expressed as mean-standard error. Arterial luminal area and wall thickness size are seen (B, C).

*P < 0.05, **P < 0.01, ***P < 0.001

Figure 3
Figure 3. Representative Tunel staining for basilar arteries at 72 hrs after subarachnoid hemorrhage (SAH) showing anti-apoptotic effects of BVZ (a) Control group (b) SAH group (c) SAH+BVZ group. Scale bar 50 μm. There were no Tunel positive staining endothelial cell nucleus (arrow) in tunica intima in control group (a). In SAH group (b), the nuclei of endothelial cells on the corrugated internal elastic lamina were stained Tunel positive. In the SAH+BVZ group, Tunel positive staining endothelial cell nuclei are seen in luminal surface. Tunel positive apoptotic endothelial cell nucleus (stained with diaminobenzidine [DAB] brown color). Normal endothelial cell nucleus (stained with methyl green). Scale Bar, 50 μm. B. Measurements of Tunel positive cells expressed as mean-standard error. **P < 0.01, ***P < 0.001.