Attenuation of cerebral vasospasm by systemic administration of an endothelin-A receptor antagonist, TBC 11251, in a rabbit model of subarachnoid hemorrhage

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Cerebral vasospasm is a major complication of subarachnoid hemorrhage (SAH) after the rupture of an intracranial aneurysm. Although the cause of cerebral vasospasm has not been fully established, several lines of evidence suggest that the vasoconstrictor peptide endothelin (ET) may play a crucial role. In the present study, the potential of TBC 11251 (TBC), a newly developed ET_A receptor antagonist, to prevent and/or reverse cerebral vasospasm was examined in a well-established rabbit model of SAH.

Sixty-five New Zealand White rabbits were assigned to one of six groups. Experimental SAH was induced in rabbits comprising five of the groups by injecting autologous arterial blood into the cisterna magna. The treatment groups were as follows: 1) control (no SAH); 2) SAH only; 3) SAH + placebo at 24 and 36 hours (24/36); 4) SAH + TBC (24/36); 5) SAH + placebo twice daily (BID); and 6) SAH + TBC BID. All drug-treated animals received an intravenous dosage of 5 mg/kg TBC. After 48 hours, the animals were killed by intracardiac perfusion with fixative. The brainstems were removed and the basilar arteries (BAs) were prepared for histological examination. The cross-sectional area of each BA was measured using computer-assisted videomicroscopy by an investigator blind to the group from which it came. A one-way analysis of variance and paired group mean comparisons with the post-hoc Fisher least significant difference test were used for analysis of BA diameters and physiological parameters.

The model provided reliable vasospasm, with the mean BA cross-sectional area constricting from 0.388 mm² in the control group to 0.106 mm² (27.4% of control) in the SAH only group. Treatment with TBC (24/36) after SAH (reversal protocol) produced a mean BA area of 0.175 mm² (44.2% of control) which, although larger than the placebo group value of 0.135 mm² (39.9% of control), was not statistically significant. However, treatment with TBC BID (prevention protocol) produced a mean BA area of 0.303 mm² (78.1% of control) compared with the placebo BID value of 0.134 mm² (34.6% of control); this effect was statistically significant (p < 0.01). There were no side effects noted and no differences in the mean arterial pressures between drug and placebo groups.

These findings demonstrate that systemic administration of the ET_A receptor antagonist TBC
significantly attenuates cerebral vasospasm after SAH when given as a preventative therapy, and they provide additional support for the role of ET in the establishment of vasospasm.

**Key Words** *cerebral vasospasm* *endothelin-1* *endothelin-A antagonist* *subarachnoid hemorrhage*

Annually in North America, 28,000 people suffer subarachnoid hemorrhage (SAH) secondary to rupture of an intracranial aneurysm. After successful surgical clipping of an identified aneurysm, the leading cause of morbidity and mortality is cerebral vasospasm. Although the cause of cerebral vasospasm is probably multifactorial, endothelin (ET)-1 is a potent peptide vasoconstrictor that has been implicated as one of the possible mediators of vasospasm after SAH.[23] Endothelin-1 causes a profound and sustained constriction of cerebral vessels[12,13,19] and is present at elevated levels in cerebrospinal fluid (CSF) after SAH.[6,8,9,14,20] In addition, it has been demonstrated in numerous animal studies that ET-directed therapy after SAH attenuates cerebral vasospasm.[2,3,5,7,10,11,15,16,18,24] A nonpeptide ET receptor antagonist, TBC 11251 (TBC), that selectively binds the ET<sub>A</sub> receptor with an affinity 6400 times that of the ET<sub>B</sub> receptor has recently been developed and has been demonstrated to be very potent in vivo. It can be administered orally.[22] In the present study the potential of this new ET<sub>A</sub> antagonist to prevent and/or reverse cerebral vasospasm after SAH was examined in a well-established rabbit model.[7]

**MATERIALS AND METHODS**

Sixty-five male New Zealand White rabbits, weighing between 3.35 and 4 kg were randomly assigned to one of six groups. Experimental SAH was induced in rabbits comprising five of these groups. Animals in treatment groups received intravenously administered drug or placebo twice daily (BID) and were killed 48 hours after SAH by perfusion fixation. All experimental protocols were approved by the University of Virginia Animal Research Committee.

In one arm of this study, prevention of vasospasm was attempted by initiating intravenous drug therapy 1 hour after SAH. Reversal of vasospasm after it had commenced was attempted by delaying treatment until 24 hours after SAH. The groups were as follows: 1) control (no SAH, 10 animals); 2) SAH only (11 animals); 3) SAH + placebo at 24 and 36 hours (24/36) (reversal arm, nine animals); 4) SAH + 5 mg/kg TBC 24/36 (reversal arm, 12 animals); 5) SAH + placebo BID (prevention arm, 11 animals); and 6) SAH + 5 mg/kg TBC BID (prevention arm, 11 animals). Animals in the prevention arm received dosages at 1, 12, 24, and 36 hours after SAH, whereas those in the reversal arm received injections at only 24 and 36 hours after SAH.

**Induction of Experimental SAH**

Animals were anesthetized by an intramuscular injection of a mixture of ketamine (40 mg/kg) and xylazine (8 mg/kg) and intubated endotracheally. Three milliliters of arterial blood was withdrawn from the central ear artery and injected using a 23 gauge butterfly needle into the cisterna magna over a 10-second period. The animals were positioned in ventral recumbency for at least 15 minutes after the injection of blood to facilitate clot formation at the ventral aspect of the brainstem. The animals were monitored closely for respiratory compromise and were artificially ventilated if necessary until spontaneous respiration resumed. They were subsequently extubated and returned to their cages when fully awake. The animals were given free access to food and water and were closely observed for
neurological deficits over the ensuing 48 hours. Two animals became hemiparetic and were excluded from further study.

**Perfusion-Fixation Protocol**

Forty-eight hours after SAH occurred, all of the animals were reanesthetized, intubated, and ventilated using a small animal ventilator (model 683; Harvard Apparatus Co., So. Natick, MA). The animals were paralyzed by means of an intravenous injection of 0.2 mg/kg pancuronium bromide. The central ear artery was catheterized to allow blood pressure monitoring and arterial blood gas analysis. The ventilation rate and oxygenation were adjusted to maintain PaO$_2$ and PaCO$_2$ within physiological ranges. After establishing satisfactory blood gas parameters, the thorax was opened and the aorta was cannulated via the left ventricle. The vascular system was perfused with 300 ml of Hank’s balanced salt solution, pH 7.4, at 37°C, followed by 500 ml of a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in Hank’s balanced salt solution, pH 7.4, at 37°C. All perfusions were performed at a pressure of 120 cm H$_2$O. After completion of the perfusion-fixation protocol, the brains were removed, placed in the same fixative solution, and stored overnight at 4°C. Two animal brains that exhibited inadequate fixation before storage were excluded from the study.

**Histological Preparation**

After fixation, the basilar arteries (BAs) were dissected from the brainstem and the proximal third of the vessel was removed. The investigator harvesting the arteries, blind to the groups from which they came, scrutinized the brainstem for the presence of thick subarachnoid clot along the course of the BA. Nine brainstems with insufficient clot along the BA were discarded. Basilar arteries that displayed vascular abnormalities (one) or were torn (one) were also discarded. The harvested BA segment was then cut into 2-mm segments and washed several times in 0.1 mol/L phosphate-buffered solution (PBS) at pH 7.4. The segments were fixed in 1% osmium tetroxide in PBS for 1 hour at room temperature and then washed again with PBS. Vessel segments were dehydrated through a graded ethyl alcohol series, followed by addition of a 1:1 mixture of propylene oxide and epoxy resin and were stored overnight. The next day, they were flat embedded in 100% epoxy resin and allowed to polymerize at 60°C for 48 hours. Cross sections of BAs were cut at a thickness of 0.5 µm with a ultramicrotome, mounted on glass slides, and stained with 0.5% toluidine blue for morphometric analyses.

**Morphometric Analysis**

The cross-sectional area of BA lumen was measured by an investigator blind to the treatment groups. Morphometric measurements of three randomly selected arterial cross sections from each BA were made using the Image 1 Analysis System (Universal Imaging, W. Chester, PA). The luminal area for each BA was obtained by averaging these three measurements.

**Statistical Analysis**

A one-way analysis of variance was performed on the entire dataset of morphometric measurements. A pairwise multiple comparisons post-hoc analysis was performed using the Fisher least significant difference test. Group data are expressed as means ± standard of the means.

**RESULTS**

**General Observations**
The physiological parameters were measured at the time of the perfusion-fixation. There were no significant differences among the groups for any of these parameters. Fifteen of the 65 animals excluded from further study came from these groups: SAH only (five), placebo (24/36) (one), placebo BID (four), TBC (24/36) (two), and TBC BID (three).

**Histological Analysis**

The BAs in the SAH only and SAH + placebo groups exhibited substantial corrugation of the internal elastic lamina. Corrugation of the internal elastic lamina was less prominent in the TBC (24/36) group and was minimal in the TBC BID group.

**Cross-Sectional Area Measurements**

The mean BA cross-sectional area for each group is represented graphically in Fig. 1. The model provided reliable vasospasm with the mean BA cross-sectional area constricting from 0.388 mm$^2$ in the control group to 0.106 mm$^2$ (27.4% of control) in the SAH only group. In the reversal protocol treatment with TBC (24/36) produced a mean BA area of 0.175 mm$^2$ (44.2% of control) which, although larger than the placebo group value of 0.135 mm$^2$ (39.9% of control), was not statistically significant. However, in the prevention protocol treatment with TBC BID produced a mean BA area of 0.303 mm$^2$ (78.1% of control) compared with the placebo BID value of 0.134 mm$^2$ (34.6% of control). The protective effect of TBC achieved statistical significance in the TBC BID group (p < 0.01). Although treatment with TBC (24/36) did not ameliorate vasospasm in a statistically significant way, a trend toward improvement was noted. There were no side effects and no differences in the mean arterial pressures between the drug and placebo groups.
Although the cause of cerebral vasospasm after SAH is probably multifactorial, mounting evidence suggests that ET-1 may play an important role in the pathophysiology of this interesting, yet devastating, disease process.[1,21,24] Several investigators have demonstrated that ET-1 is elevated after SAH in both the blood and CSF of patients and laboratory animals. Cocks, et al.,[4] elegantly demonstrated that when oxyhemoglobin is present in tissue culture media, it can increase the production of ET-1 by endothelial cells. This study has been confirmed by Ohlstein and Storer.[17] Because ET-1 is an extremely potent vasoconstrictor, numerous studies have been performed to confirm its role in cerebral vasospasm and to assist in the design of therapeutic strategies to block its production or effects on the cerebral vasculature.

Although several groups of investigators have attempted to decrease ET production after SAH by using RNA synthesis inhibitors[21] or ET-1 antisense techniques,[18] most studies have been concentrated on the use of ET-converting enzyme inhibitors or ET antagonists.[2,11] Matsumura, et al.,[15] were the first...
to demonstrate that an ET-converting enzyme antagonist could partially prevent cerebral vasospasm in a canine SAH model. This approach has subsequently been used by other groups, with excellent results.[2,5] More recent studies have focused on the use of ET_{A/B} or ET_{A} receptor antagonists to prevent or reverse cerebral vasospasm in several animal models.[3,5,10,11,16,24] Although the results have been variable, virtually all studies have demonstrated less vasoconstriction in animals treated with ET antagonists.

It is still unclear whether ET_{A} or ET_{A/B} receptor antagonists will be the most effective in the treatment of cerebral vasospasm. Zuccarello, et al.,[24] elegantly demonstrated that ET_{A} and ET_{A/B} receptor antagonists were similarly effective in preventing spasm in a rabbit double-SAH model. However, because ET_{B} antagonists may prevent a naturally occurring vasodilating response by blocking endothelial cell ET_{B}-mediated release of nitric oxide, a specific ET_{A} antagonist may be preferable.[24] Clinical trials of both specific and nonspecific ET antagonists in humans will need to be performed to determine the role of the ET_{B} receptor in the treatment of vasospasm.

In the present study, the ET_{A} receptor antagonist TBC has been shown to attenuate SAH-induced vasospasm in a significant and substantive fashion. Although the amount of vasospasm 24 hours after SAH was partially reversed by TBC, the improvement was not statistically significant. Because only one dose (5 mg/kg) level was used in this study, it is possible that a higher dose would be more effective in reversing cerebral vasospasm. The TBC was well tolerated by the animals and they maintained stable blood pressures. There were no observable untoward effects.

CONCLUSIONS

In summary, twice daily intravenous administration of TBC 11251 to rabbits after induction of experimental SAH significantly and substantially prevented vasospasm in a random, blinded, placebo-controlled trial. These results support the role of ET-1 in the pathogenesis of cerebral vasospasm. Further studies are warranted to evaluate the oral efficacy of TBC 11251, to establish the dose-response characteristics of the drug, to evaluate the CSF and serum levels of the drug and its metabolites, and to investigate whether higher drug doses could reverse cerebral vasospasm after it is established. This study, as well as many others, strongly support the investigation of ET_{A} antagonists in human clinical trials aimed at preventing cerebral vasospasm.

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References


3. Clozel M, Watanabe H: BQ-123, A peptidic endothelin ET_{A} receptor antagonist, prevents the early


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