JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2020; 4:310-317
https://doi.org/10.30621/jbachs.2020.1125

Original investigation

Berberine Attenuates Cerebral Vasospasm After Experimental Subarachnoid Hemorrhage Via Modulating AMPK/Rho Pathway

Ozge Altintas Kadirhan[1], Meltem Kumas[2], Ersin Karatas[3], Hasan Serdar Mutlu[4], Abdurrahim Kocyigit[3], Talip Asil[5]

[1]Beykent University, Faculty of Medicine, Department of Neurology, İstanbul, Turkey
[2]Dokuz Eylul University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Izmir, Turkey
[3]Bezmialem Vakıf University, Facult of Medicine, Department of Medical Biochemistry, istanbul, Turkey
[4]Istanbul University, Istanbul Medical Faculty, Department of Histology and Embryology , istanbul, Turkey
[5]Bahcelievler Memorial Hospital, Neurology Clinic, İstanbul , Turkey

Address for Correspondence: Meltem Kumaş, **E-mail:** kumasmeltem@gmail.com
Received: 04.05.2020; **Accepted:** 17.06.2020; **Available Online Date:** 15.10.2020

Cite this article as: Altintas Kadirhan O, Kumas M, Karatas E, Mutlu HS, Kocyigit A, Asil T. Berberine Attenuates Cerebral Vasospasm after Experimental Subarachnoid Hemorrhage via Modulating AMPK/Rho Pathway. J Basic Clin Health Sci 2020; 4:310-317.

ABSTRACT

Purpose: Our goal is to clarify the effectiveness of berberine (BBR) on cerebral vasospasm induced by subarachnoid hemorrhage.

Methods: Thirty male Sprague-Dawley rats (350–400 g) were randomly allocated to five groups (sham group, SAH, BBR, SAH+BBR1 or SAH+BBR2. Experimental SAH model was induced by applying autologous blood into the cisterna magna at interval of 48 hours. To evaluate early and late effects of BBR, we allocated BBR treated groups as SAH+BBR1 and SAH+BBR2 (respectively, received BBR at a dose of 20 mg/kg 15 minutes and 6 hours after first SAH induction). Rats were sacrificed on 72-hour after the study onset. Cross-sections of basilar artery was investigated by histologically. Total antioxidant status (TAS) and total oxidant status (TOS) of brain tissue were studied by spectrophotometric assay. Oxidative stress index (OSI) was calculated. NAPPH Oxidase 4 (NOX4) enzyme levels were measured by ELISA method. Endothelial nitric oxide synthase (e-NOS), phosphorylated e-NOS (pe-NOS), AMP-activated protein kinase (AMPK), phosphorylated AMPK (pAMPK), Rho kinase and cingulin protein expressions were detected by Western blot analysis.

Results: SAH+BBR1 and SAH+BBR2 groups significantly demonstrated lower OSI values, increased basilar artery cross-sectional luminal area in comparison with the SAH group. Increased Phosho-eNOS, eNOS, P-AMPK levels and Cingulin expression, decreased Nox4 and Rho-kinase levels were shown in BRB treated SAH groups relative to the SAH group.

Conclusion: Berberine might be a neuroprotective agent to improve impaired cerebrovascular spasm.

Keywords: berberine, subarachnoid hemorrhage, cerebral vasospasm, AMP-Activated Protein Kinase, Rho kinase, endothelial dysfunction

INTRODUCTION

Onset cerebral edema and vasospasm after subarachnoid hemorrhage predict unfavorable neurologic outcome and death (1). Endothelial dysfunction is the leading vascular factor to trigger cerebral edema and vasospasm (2, 3). Cerebral edema and vasospasm develop respectively in response to increased microvascular permeability and released intraluminal acting spasmogens.

Dynamic control of the endothelial barrier mainly involves complex signaling mechanisms of tight junction proteins (TJs) (4, 5). Cingulin has been newly investigated a key component of the multiprotein complex on the cytoplasmic face of the TJs (4, 6). Recent studies have indicated that RhoA associated kinase (ROCK) inhibitors could reverse cerebral vasospasm after SAH (3, 7). Cingulin regulates the activity of the RhoA/ROCK pathway (4, 8). Also, interactions of junctional and cytoskeletal proteins were mediated by cingulin, through its adenosine monophosphate-activated protein kinase (AMPK)-dependent phosphorylation (4, 9).

Berberine (BBR), is an alkaloid extract of many medicinal plants, has been using as a traditional medicine. Berberine could cross the blood-brain barrier directly and could be transported into the neurons in a concentration-dependent and time-dependent manner (10, 11). The data from animal and clinical investigations suggested that berberine (BBR) could be an anti-hypertensive, anti-atherosclerotic and vasculoprotective agent possibly by

activating adenosine monophosphate-activated protein kinase (AMPK) (12, 13). Indeed, AMPK may play an essential role to regulate endothelial nitric oxide synthesis (14). Therefore, the suppressive modulation of BBR on RhoA pathway is investigated in association with its anti-oxidative effect (12).

On the basis of these findings, we aimed to analyze the endothelial role of BBR whether it could attenuate cerebral vasospasm by controlling AMPK/RhoA pathways.

MATERIAL and METHODS

Animals

The Experimental Animal Research Laboratory of BezmiAlem Vakif University approved the experimental study protocol (Istanbul, Turkey). All procedures were carried out in keeping with institutional guidelines. All animals were housed at room temperature (22–25°C) under a diurnal (12:12-h day/night) cycle with free access to food and water during the experiment. An infrared thermometer (Nimomed®) was used to control all animals' body temperature. Weights and glucose concentrations of animals were measured three times such as initial, before and after the SAH induction.

Study Design

Thirty male Sprague-Dawley rats (350–400 g) were divided into following groups: Sham group (n=6), SAH (n=6), BBR (n=6), SAH+ BBR1 (n=6) or SAH+ BBR2 (n=6). Subarachnoid hemorrhage was induced by a double injection of autologous blood into the cisterna magna as described by Dudhani et al (15). The second SAH model was performed 48 hours after the first SAH model.

The induction of SAH by double-injection method autologous blood in rats

Other studies have shown that second injections of blood 24 hr apart can induce a more significant degree of vasospasm we believe it would not mimic the time course of vasospasm in humans where vasospasm rarely occurs prior to day three after SAH (15). In order to more closely mimic this time course we made the second injection of blood 48 hr after the first injection. Low mortality rates allow for a more thorough understanding of the entire mechanism of cerebral vasospasm.

Briefly, animals were anesthetized with ketamine (4 mg/100 g) and xylazine (1.5 mg/100 g) by intramuscular injection and placed on an operation plate in the supine position. Their heads and limbs were fixed. Using an electronic shaver a neck to nose area of hair around the sub-occipital region is shaved. Firstly, the tail artery is catheterized and 0.25 ml of arterial blood was withdrawn into a syringe. Using a vertical midline access with 26-gauge catheter, we accessed to the cisterna magna through vertical midline line. Once identified a 26 gauge needle was inserted into the cisterna magna, 0.25 ml of cerebrospinal fluid (CSF) was withdrawn into a syringe to avoid increased intracranial pressures with injection of autologous blood volume. And then the 0.25 ml of blood extracted from the tail artery was injected slowly into the cisterna magna. The animal was placed prone on a warming surface with a 30° head down position for 15 min to allow blood to congeal in the cisterns around the basilar artery. The second surgery was performed 48 hr apart from first surgery. The sham operation consisted of the same manipulation but without injection of the autologous blood.

Berberine chloride (BBR) was purchased from Sigma-Aldrich, Germany. Zhou et al. converted a commonly used dosage of BBR (2 mg/kg) in clinical practice as the chosen dose 20 mg/kg in our study (16). Animals were admistered intraperitoneal injection of BBR through 30 min. Berberine was dissolved in distilled water; because of that we did not allocate a vehicle group to the study (16).

In a rat double-hemorrhage model, a biphasic vasospasm was confirmed as spasm was observed 30 min. after the bleed (acute phase), and again at 48 hours (chronic phase) (1). Upon induction of SAH, pathological changes occurred within the endothelia during acute (1/2 to 4 hours) and chronic (48–72 hours) stages of vasospasm (17, 18). Furthermore, another important patho-morphological finding, the disruption of inter-endothelial tight junctions, was also observed 30 min. after hemorrhage (19). On the other hand, Osuka et al. showed for the first time a chronological change of induction of inducible NOS, detected from 6 h and peaking at 2 days after a single hemorrhage SAH (20). On behalf of these data, the study animals were admistered berberine at 15th minute (early treatment group, SAH+ BBR1 (n=6)) or at 6th hour (late treatment group, SAH+ BBR2 (n=6)) after inducing subarachnoid hemorrhage. And also, rats were sacrificed at 72th to demonstrate early brain injury (EBI) period.

Berberine administration

Database from the SAH experimental studies, the cerebral vasospasm settles in a major two-peak interval. The initial peak occurs at a hyper acute phase between 30 minutes and four hours, whereas the second peak occurs between 24th and 72th hours at a late phase after onset SAH. In order to determine early and late effects of BBR against SAH damage, we allocated BBR treated groups as SAH+BBR1 and SAH+BBR2. Both groups received BBR at a dose of 20 mg/kg intraperitoneally. BBR was administered to the SAH+BBR1 group within 15 minutes after first SAH induction and to the SAH+BBR2 group at 6th after first SAH induction.

Neurological deficit assessment

Every 24th hour, an observer blindly calculated the neurologic examination using a neurological scoring system (21).

Morphometric method of basilar artery analysis

Basillary arter was fixed in 10% formalin solution. Five-micrometer (μm) sections obtained from paraffin blocks were stained with Mayer Hematoxylin (Merck Catalog no. 05–06004/L, Milano, Germany) and Shandon Eosin Y Alcoholic (Thermo scientific, Catalog no. 6766007, Kalamazoo, MI, USA),

Basilar artery wall thickness and the cross-sectional luminal areas of the basilar artery were measured using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1 c camera, and Nikon NIS

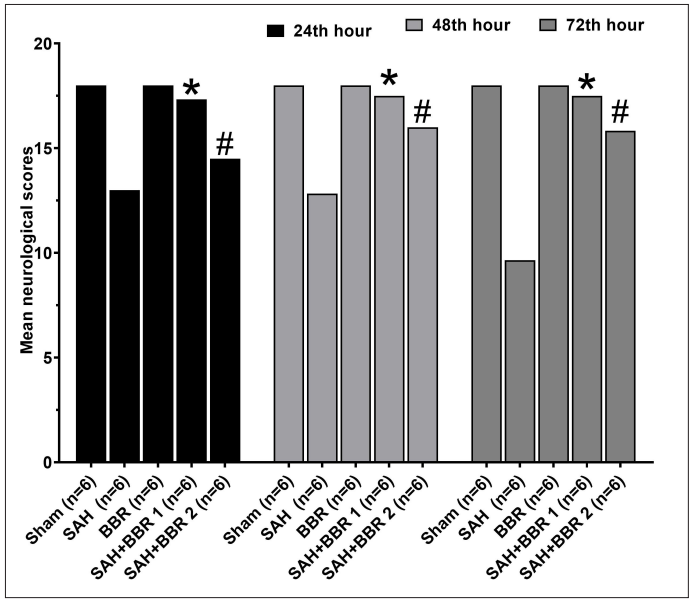

Figure 1. The mean neurological scores of the groups at 24th, 48th, 72th hours during the study period. (One-way ANOVA, post-hoc Tukey HSD) [*SAH+BBR1 group vs. SAH group (p=0.018, at 24th hour); *SAH+BBR1 group vs. SAH group (p=0.013, at 48th hour); *SAH+BBR1 group vs. SAH group (p=0.003, at 72th hour); #SAH+BBR2 group vs. SAH group (p=0.002, at 24th hour); #SAH+BBR2 group vs. SAH group (p=0.013, at 48th hour); #SAH+BBR2 group vs. SAH group (p=0.001, at 72th hour)]

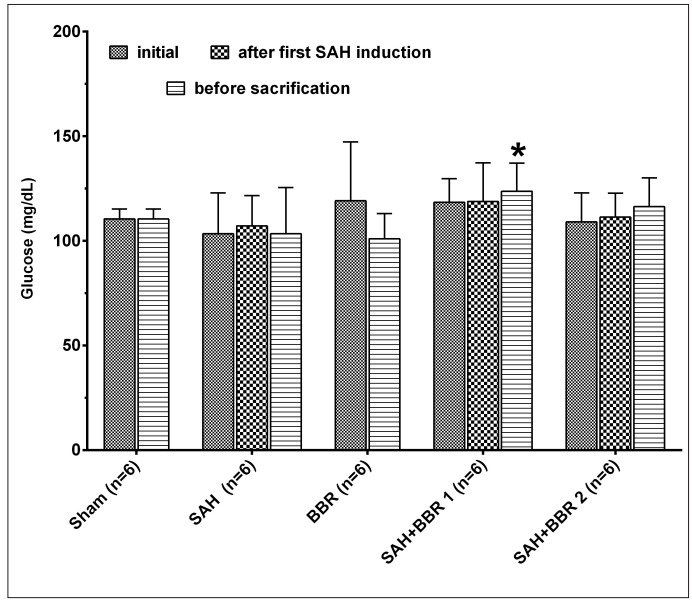

Figure 2. The mean blood glucose levels (mg/dl) of the groups measured at different periods (One-way ANOVA, post-hoc Tukey HSD; *SAH+BBR1 group vs. SAH group, p=0.021)

Elements version 4.0 image analysis systems (Nikon Instruments Inc., Tokyo, Japan) in each section in order to estimate the cerebral vasospasm.

Biochemical analyses

Total antioxidant status (TAS; mmol trolox equiv/L) and total oxidant status (TOS; (μmol H2O2 equiv/L) of brain tissues were measured in according to manufacturer's instruction. All kits were purchased commercially from Rel Assay Diagnostics; Mega Tip, Gaziantep, Turkey. The oxidative stress index (OSI) was calculated using the equation: TOS/TAS.

Western Blot analyses

Brain samples were homogenized in RIPA buffer containing proteinase inhibitor cocktail (Merck, KGaA, Darmstadt, Germany). The homogenates were centrifuged (15000 X g) at 4°C for 10 min. The total protein amount was measured according to the Bradford Coomassie brilliant blue dye method. The protein samples were separated by 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Santa Cruz Biotechnology, Texas, USA). All membranes were blocked with 5% powdered skim milk for 1 h, incubated overnight with an IgG-purified AMPKa, Phospho-AMPKa, eNOS, Phospho-eNOS, Anti-Rho, Cingulin, Anti-NOX4 antibodies (Abcam, Cambridge, United Kingdom), and then conjugated with a peroxidase-conjugated goat anti-rabbit IgG.

The expression levels of proteins were quantified by using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Piscataway, New Jersey, USA) and the bands captured with an imaging system (Vilber Lourmat Sté, Collégien, France).

Statistical analysis

Shapiro–Wilk normality test, determined normality of all data. Parametric test One-way ANOVA (post-hoc: Tukey's HSD) was preferred to multiple comparisons of normally distributed data [Shapiro–Wilk normality test, p≥0.05]. P≤0.05 was approved as statistically significant. Data was normally distributed expressed as mean ± standard deviation. All statistical analyses and bar charts were performed with SPSS 20.0 (IBM, New York, USA), and Graph Pad Prism 6.

RESULTS

General characteristics of the study groups

Neurological scores (NSs) were assessed at 24th, 48th and 72nd hours in the course of the experiment. None of the animals were death.

The mean NSs of the SAH+BBR1 group at 24th, 48th, and 72nd hours were lower than that of the SAH group (p<0.05, for all).

The mean NSs at 24th, 48th and 72nd hours in the SAH+BBR2 group were detected statistically significant according to those of the SAH group (p<0.05, for all), while NS at 72nd hr of SAH+BBR2 group was found markedly lower than SAH group (p=0.003).

Interestingly, the neurological scores were not significantly correlated between the SAH+BBR1 and SAH+BBR2 groups during study period (p>0.05) (Figure 1).

According to these results, BBR administration at 15 minutes markedly improved neurological scores assessed at all periodic times. Remarkable improvement was detected only at 72 hours after SAH induction in the SAH+BBR2 group.

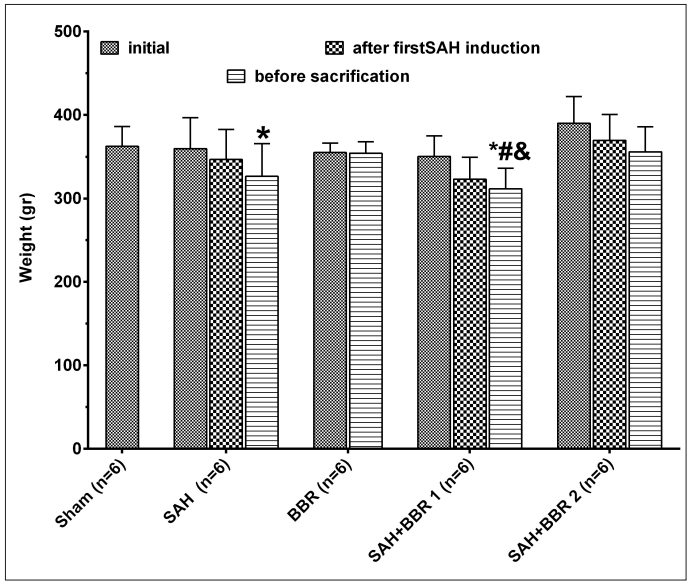

Figure 3. The mean body weight (gr) of the groups measured at different periods (One-way ANOVA, post-hoc Tukey HSD, *Sham group vs. SAH group, p=0.033; #SAH+BBR1 group vs. SAH+BBR2 group, p=0.010).

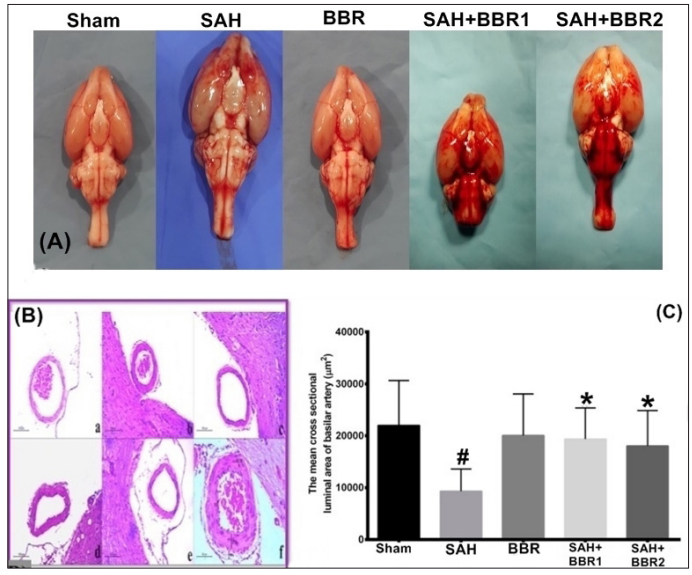

Figure 4. a–c. Gross examination of rat brain is seen **(a)**. Histologic examination of basilar arteries **(b)** (Transverse section, H-E stain, Magnification 20x; a, Sham group; b, SAH group; c, BBR group; d, SAH+BBR1 group; e, SAH+BBR2 group; f, SAH group). The mean cross sectional luminal areas of basilar artery (µm2) in all groups **(c)** (One-way ANOVA, post-hoc Tukey HSD, # Sham group vs. SAH group, p<0.001; *SAH+BBR1 group vs. SAH group, p=0.02; *SAH+BBR2 group vs. SAH group (p=0.04).

Both blood glucose levels and body weights were measured before and after SAH induction in all groups. There were not any changes between the initial and last glucose levels and body weights measurements (p>0.05, for all comparisons, Figures 2 and 3, respectively).

Despite the SAH+ BBR2 group showed similar the mean glucose levels with other groups (p>0.05), SAH+BBR1 group had markedly lowered the mean glucose levels compared to that of Sham group before sacrification (p=0.021). We observed that SAH induction dramatically reduced the body weight of rats compared to the sham group (p=0.033). On the other hand, BBR treatment had no significant effect on body weight correlated to the non-treatment groups (p>0.05). According to time-course effects of berberine, the reduction on body weight of the SAH+ BBR1 group was much greater than that of the SAH+ BBR2 group (p=0.010).

Morphometric analysis of Basilar arteries

Total basilar arterial wall thickness and cross-sectional luminal areas were measured in order to estimate the cerebral vasospasm.

On gross examination of rat brains, extensive diffuse coagulated blood present over the hemispheres (Figure 4a).

Microscopic examinations showed that endothelial dysfunction, twisting of the internal elastic lamina, and smooth muscle necrosis in the Basilar arteries (BAs) of SAH with/out treatment with berberine (Figure 4b).

The mean cross-sectional area of BA was markedly lower in SAH group than that of the sham group (p<0.001). In BBR treated groups, the mean cross-sectional luminal area of BA was higher than that of the SAH group (p=0.02 for SAH+BBR1 vs. sham group; p=0.04 for SAH+ BBR2 vs. sham group, Figure 4 c).

According to these results, we might suggest that berberine mediated pathways prevent narrowing of major cerebral arteries induced by SAH in this rat model.

Assessment of oxidative stress parameters

The lowest mean TAS and the highest TOS were detected in the SAH group; but statistical differences were not detected between groups for both TAS and TOS values (SAH vs. other groups, p>0.05, for all comparison). The mean TOS markedly lowered in SAH+ BBR1 and SAH+BBR2 groups compared to the SAH group (p=0.033 and p=0.049, respectively). The mean TAS was higher in SAH+BBR1 and SAH+BBR2 groups than that of SAH group (p>0.05, for both comparison). The highest OSI value was calculated in the SAH group correlated to other study groups (p<0.001). BBR treated groups had lower OSI value than that of SAH group (SAH+ BBR1 vs. SAH p=0.007 and SAH+ BBR2 vs. SAH p=0.015). Both SAH+BBR1 and SAH+ BBR2 groups had similar TAS and TOS values; we did not obtain any alteration in a time-course manner after berberine treatment (p>0.05). According to our results, subarachnoid hemorrhage resulted decrease in the antioxidant response and berberine treatment improved antioxidant response via decreasing TOS. Bar graphs of TAS, TOS and OSI were represented in Figure 5.

Assessment of Berberine-mediated attenuation of Cerebral Vasospasm

This study aimed to reveal the potential mechanisms related to berberine effect on improving imbalanced endothelial vasodilatation due to eNOS, NOX4, AMPK/RhoA pathway.

Firstly, to clarify the potential mechanisms on vasospasm mediated by berberine treatment, the expressions of eNOS and

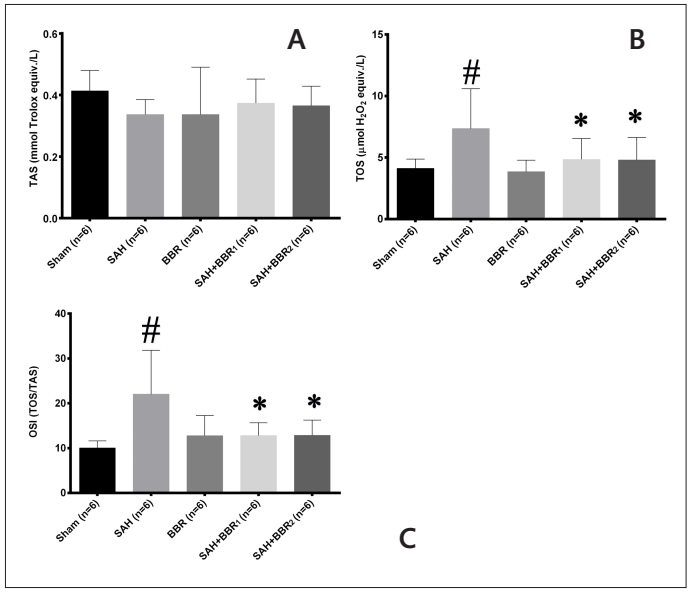

Figure 5. a–c. Assessment of oxidative stress parameters in the groups. The mean TAS levels in the groups **(a)**. The mean TOS levels in the groups **(b)** (#Sham vs. SAH group, p=0.02; *SAH+BBR1 group vs. SAH group, p=0.033; *SAH+BBR2 group vs. SAH group, p=0.049; One-way ANOVA, post-hoc Tukey HSD). The mean OSI values in all groups **(c)** (# Sham vs. SAH group, p<0.001; *SAH+BBR1 group vs. SAH group p=. 007; *SAH+BBR2 group vs. SAH group, p=0.015).

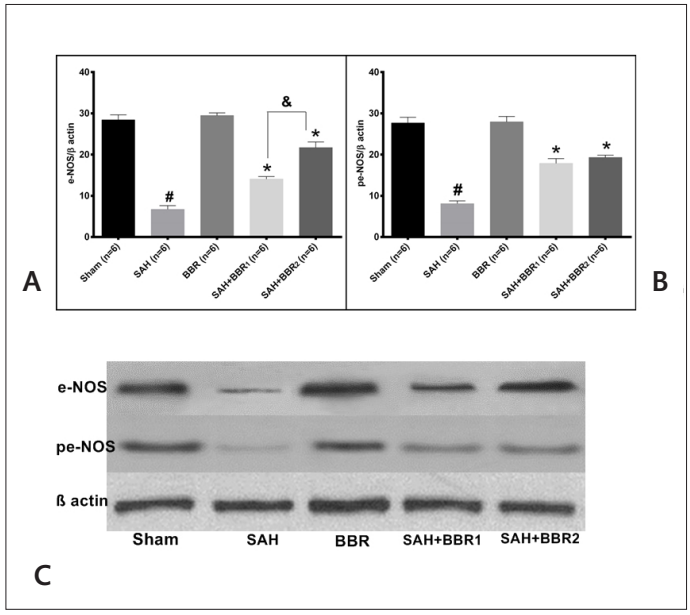

Figure 6. a–c. The mean e-NOS and pe-NOS protein expressions in the groups **(a, b)** (One-way ANOVA, post-hoc Tukey HSD; *SAH+BBR1 group vs. SAH group, p<0.0001; *SAH+BBR2 group vs. SAH group (p<0.0001). Western blot analysis of e-NOS and pe-NOS protein expressions of the groups **(c)**.

phosphorylated eNOS (peNOS) were examined. Phosphorylation of eNOS is related with eNOS activity. When compared to sham group, eNOS and peNOS expressions markedly decreased in SAH group. (p<0.0001, for both comparisons). Berberine treatment significantly increased both eNOS and peNOS expressions in the SAH+ BBR1 and SAH+ BBR2 groups compared to the SAH group (p<0.0001, for all comparison; Figure 6a and 6b, respectively). Furthermore, BBR treatment at 6th hr increased eNOS and peNOS levels more than those of BBR treatment at 15 minutes after induction of SAH (SAH+ BBR1 vs. SAH+ BBR2, p<0.0001, Figure 6 c). Time-course effect of berberine therapy might prevent imbalanced endothelial vasodilatation due to increasing eNOS expression after receiving the therapy at hyperacute phase of SAH onset.

The level of NOX4 markedly was increased in the SAH group than that of the sham group (p<0.0001). NOX4 expressions in SAH+BBR1 and SAH+BBR2 groups were markedly decreased compared to that of the SAH group (p=0.001 and p<0.0001, respectively). Indeed, we could recommend that berberine prevent or reduce oxidative stress by lowered NOX4 expression (Figure 7).

While the mean protein expression levels of AMPK were not significantly significant among groups (Figure 8a), the protein expression levels of phosphorylated AMPK markedly increased in SAH+ BBR1 and SAH+ BBR2 groups compared to the SAH group (p<0.01 and p<0.0001, respectively; Figure 8b and 8 c). The phosphorylation of AMPK by berberine suggests direct interaction between the effect of berberine and the AMPK signaling pathway.

Rho-kinase significantly higher expressed in the SAH group compared to the Sham group (p<0.0001), while the level significantly lower expressed after berberine treatment in SAH+ BBR1 and SAH+ BBR2 groups (p<0.0001, p=0.001, respectively; Figure 9a and 9b).

As a result, we could suggest that berberine treatment take place in intense vasoconstriction by increasing peNOS and phosphorylated-AMPK expressions and decreasing ROCK activity after subarachnoid hemorrhage.

Assessment of Microvascular Permeability

The expression of cingulin in the SAH group was markedly lower than that of the sham group (p<0.0001). Berberine treatment resulted in a decrease of cingulin expression in SAH+BBR1 and SAH+BBR2 groups (p=0.001, SAH+ BBR1 vs. SAH; p<0.0001, SAH+ BBR2 vs. SAH; Figure 10a and 10b).

It may be suggested that increasing cingulin expression in the SAH group could be the reason for vascular endothelial disruption. We also proposed that berberine as a time-course manner could have a possible neuroprotective effect against brain damaged as a result of subarachnoid hemorrhage.

DISCUSSION

According to the current database, subarachnoid hemorrhage has a multifactorial pathologic pathway, especially including critical intracranial arterial narrowing thereby causes neurologic deficit and death. The researches have suggested the vasculo-neuronal-glial triad model to clarify the pathologic pathways causing cerebral vasospasm and edema. The Vasculo-Neuronal-Glia triad model is more suitable and significant rather than the

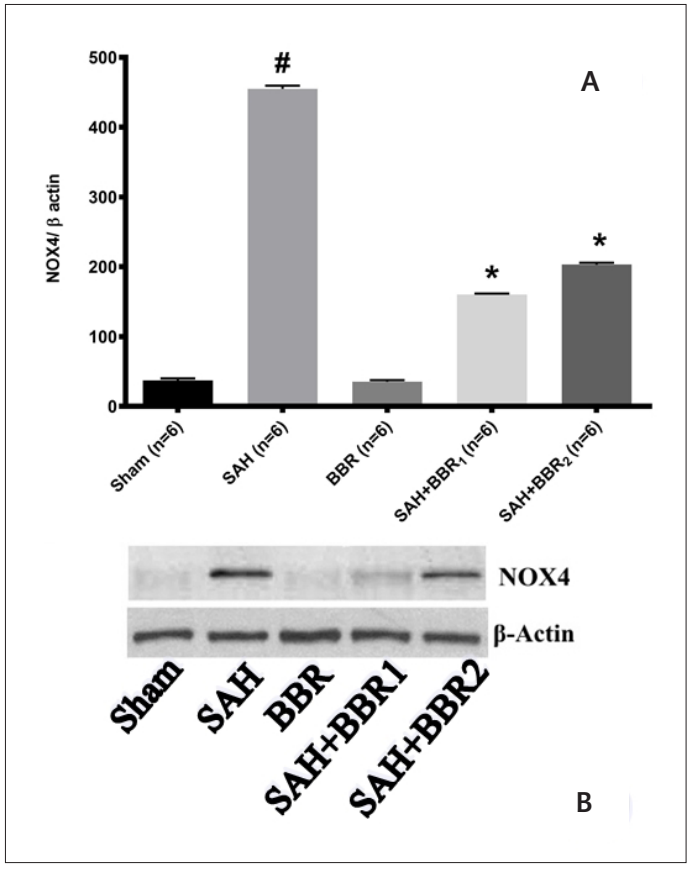

Figure 7. The mean NOX4 enzyme levels in the groups (# SAH group vs. Sham group p<0.0001; * SAH+BBR1 group vs. SAH group, p=0.001; * SAH+BBR2 group vs. SAH group, p<0.0001; One-way ANOVA, post-hoc Tukey HSD)

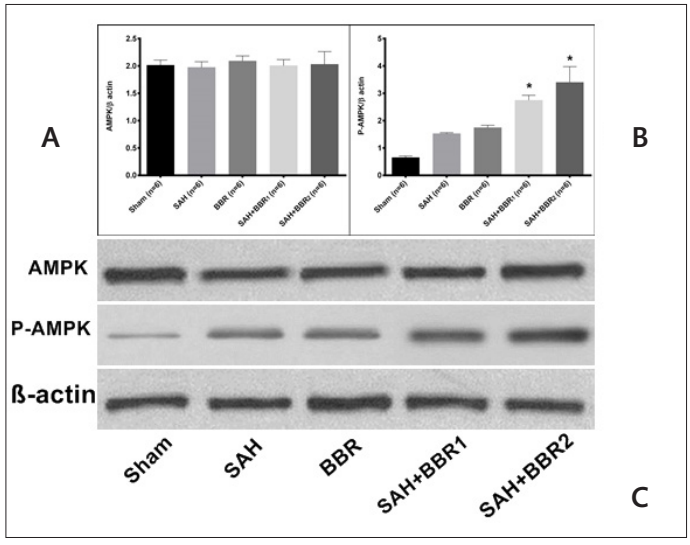

Figure 8. a–c. The mean AMPK and P-AMPK protein expressions in the groups **(a, b)** (*SAH+BBR1 group vs. SAH group, p<0.0001; *SAH+BBR2 group vs. SAH group (p<0.0001; One-way ANOVA, post-hoc Tukey HSD). C. Western blot analysis of AMPK and p-AMPK protein expressions of the groups **(c)**.

neurovascular unit model due to consist of all cerebral structures as neurons, glial cells, astrocytes, perivascular nerves, vascular endothelial cells providing constant cerebral blood flow under physiological and pathological conditions (22).

The immediate neuropathogical injury named as early brain injury (EBI) arises in the first three days after SAH. Otherwise, delayed and progressive brain injury (DBI) consists of cerebral vasospasm and cortical spreading ischemia appeared within 4–14 days after SAH (18). In the present study, we performed SAH experimental model to estimate the neuroprotective effectiveness of berberine treatment against early brain injury (EBI) on whether BBR administration improves cerebral vasospasm, oxidative stress, vascular endothelium disruption, and neurological deficits.

Acute cerebrovascular spasm is caused by imbalance of endothelial dependent– vasodilatation and vasoconstriction after SAH (1, 3). The endothelial nitric oxide synthase (eNOS) was generated NO in the intracranial artery endothelial cells. NO is a key messenger molecule at the regulation of the different pathways in the central nervous system, however; excessive production of NO can cause neuronal cell damage and death (23). AMPK induces eNOS activity to generate NO synthesis (23, 24). Besides, AMPK activity could enhance under hypoxic, ischemic conditions to regulate cerebral angiogenesis and neurogenesis (25). Osuka et al. found that in a single-hemorrhage model, both eNOS and AMPK activity was significantly induced in the rat basilar arteries at a hyperacute interval after SAH onset (20). In our study, after BBR treatment the levels of eNOS and P-AMPK were significantly increased, implying that the AMPK-eNOS signaling pathway could participate to regulate impaired cerebral blood flow because of vasospasm.

Furthermore, the increased level of reactive oxygen species (ROS) in the cerebrospinal fluid has synchronously generated with cerebral vasospasm (3, 26). Nox4 (NADPH Oxidase 4) is especially highly expressed in neuronal and endothelial cell types (26, 27) generating ROS as a mitochondrial enzyme. Concurrently, SAH-induced oxidative stress enhances NO depletion and reduces the bioavailability of NO (28). Thus, ROS has been shown to induce vascular contraction through activation of the RhoA pathway (29). Pharmacological inhibition of reactive oxygen forming in neuronal and endothelial cells has been shown to inhibit the programmed cell death and improve the blood-brain barrier (BBB) permeability (30). Won et al. suggested that Apocynin as a Nox inhibitor improved the impaired blood-brain barrier integrity and cytotoxic edema as a result of receiving thrombolytic agents (31). Besides, elevated Nox4 expression in peri-infarct and contralateral brain region after stroke demonstrated that Nox4 could be trigger neuroprotective pathways induced by ischemia (20, 32) Indeed, our data showed that elevated Nox4 and oxidative stress expression were lowered by berberine administration. In view of this, after more detailed data, Nox4 might serve as a next generation pharmacological agent against ischemic injury.

Paracellular diffusion is limited by tight junctions between microvascular endothelial cells. Junctional RhoA activation

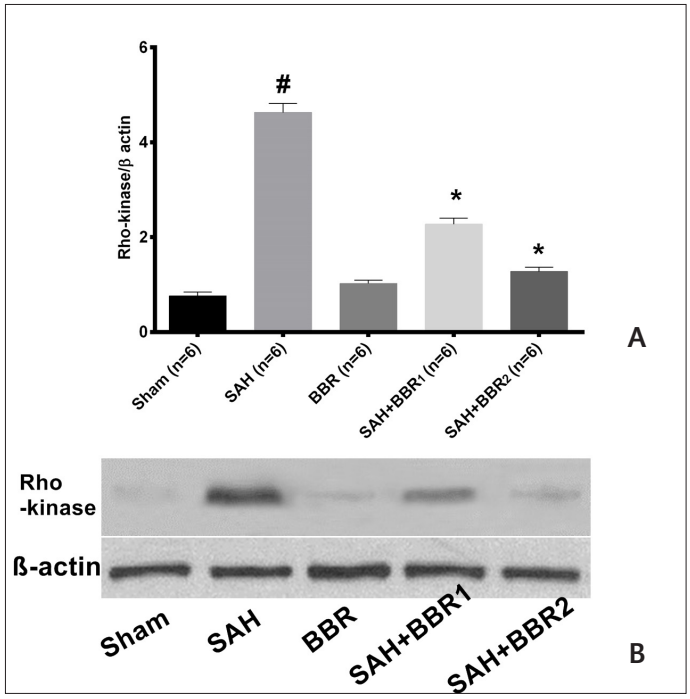

Figure 9. a, b. The mean Rho-Kinase protein expression of the groups **(a)** (*SAH+BBR1 group vs. SAH group, p<0.0001; *SAH+BBR2 group vs. SAH group, p<0.0001; One-way ANOVA, post-hoc Tukey HSD). Western blot analysis of Rho-kinase protein expression of the groups **(b)**.

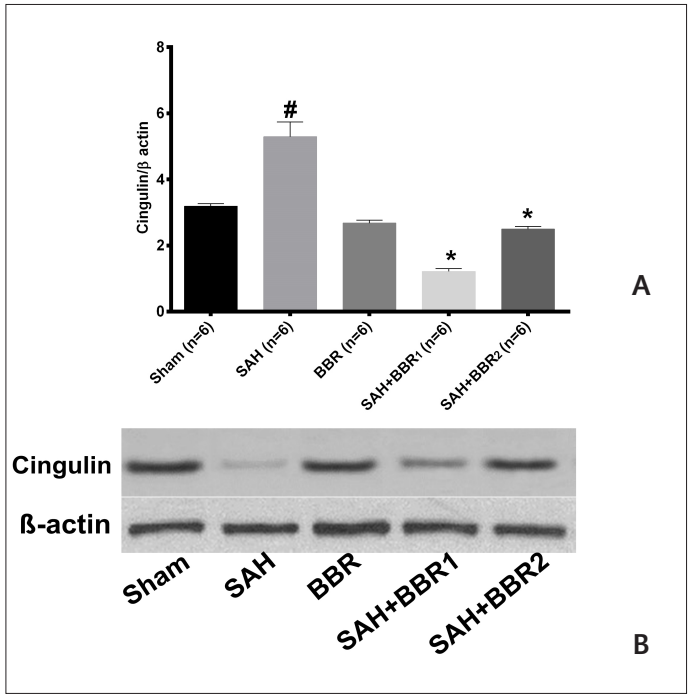

Figure 10. a, b. The mean cingulin protein expression of the groups **(a)** (*SAH+BBR1 group vs. SAH group, p<0.0001; *SAH+BBR2 group vs. SAH group, p<0.0001; One-way ANOVA, post-hoc Tukey HSD). B. Western blot analysis of cingulin protein expression of the groups **(b)**.

support junction main formation and regulate cytoskeleton-mediated processes. The linkage between junctional proteins and cytoskeleton plays a crucial role to regulate the vascular-junction dynamics and tissue remodeling (5, 6, 8). Yano et al. recently investigated that the interaction between the cytoskeleton and junctional proteins was mediated by cingulin, through its AMP-dependent phosphorylation (4). Cingulin depletion trigger RhoA activity thereby these factors disrupt vascular permeability (8). Indeed, RhoA pathway and Cingulin play a crucial role in sustained cerebral vasospasm and endothelial injury after onset SAH. Fasudil is a Rho-kinase inhibitor agent that can be received by acute SAH patients in order to attenuate cerebral vasospasm (33). We found that ROCK activity was decreased by berberine treatment in SAH-induced rats, indicating that suppressive mechanisms might occur to reverse RhoA activity. Also, we showed that berberine treatment prevented BBB breakdown, improved neurological outcome due to preserve cingulin expression and reduce oxidative markers.

CONCLUSION

As a result, in this study, key mechanisms of berberine as a neuroprotective agent could be explainable due to attenuation smooth muscle hypercontraction by inhibition of RhoA/ROCK pathway, upregulation of eNOS expression-activity by AMPK phosphorylation, reduction of oxidative responses and improve vascular endothelial function by preventing Cingulin depletion. We could also suggest that these data supported the efficacy of berberine treatment for subarachnoid hemorrhage when administered within 6 hours of symptom onset. These findings, together with the observations of previous studies, raise the possibility that berberine might be effective prophylaxis to prevent endothelial dysfunction associated with SAH.

Informed Consent: Current study is an experimental study

Compliance with Ethical Standards: Study procedure was approved by the Animal Care and Use Committee at Bezmialem Vakif University and performed in accordance with institutional guidelines (Decision no: 2015-60)

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - OAK, MK; Design - OAK, MK; Supervision - OAK, MK; Fundings - TA; Materials - TA, MK; Data Collection and/or Processing - MK, EK, HSM; Analysis and/or Interpretation - OAK, MK; Literature Search - OAK, MK; Writing Manuscript - OAK, MK; Critical Review - AK, TA

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

Presented In: This study was accepted as an oral presentation at 52nd National Neurology Congress 2016, Antalya, Turkey

REFERENCES

1. Cahill J, Calvert JW, Zhang JH. Mechanisms of early brain injury after subarachnoid hemorrhage. J Cereb Blood Flow Metab 2006;26:1341–1353. [CrossRef]
2. Park S, Yamaguchi M, Zhou C, Calvert JW, Tang J, Zhang JH. Neurovascular protection reduces early brain injury after subarachnoid hemorrhage. Stroke 2004;35:2412–2417. [CrossRef]
3. Archavlis E, Carvi Y, Nievas M. Cerebral vasospasm: a review of current developments in drug therapy and research. J Pharm Technol Drug Res 2013;2:1–18. [CrossRef]
4. Yano T, Matsui T, Tamura A, Uji M, Tsukita S. The association of microtubules with tight junctions is promoted by cingulin phosphorylation by AMPK. J Cell Biol 2013;203:605–614. [CrossRef]
5. Spindler V, Schlegel N, Waschke J. Role of GT Pases in control of microvascular permeability. Cardiovasc Res 2010;87:243–253. [CrossRef]
6. Citi S, Pulimeno P, Paschoud S. Cingulin, paracingulin, and PLEKHA7: signaling and cytoskeletal adaptors at the apical junctional complex. Ann N Y Acad Sci 2012;1257:125–132. [CrossRef]
7. Fujii M, Duris K, Altay O, Soejima Y, Sherchan P, Zhang JH. Inhibition of Rho kinase by hydroxyfasudil attenuates brain edema after subarachnoid hemorrhage in rats. Neurochem Int 2012;60:327–333. [CrossRef]
8. Terry S, Nie M, Matter K, Balda MS. Rho signaling and tight junction functions. Physiology (Bethesda) 2010;25:16–26. [CrossRef]
9. Ronnett GV, Ramamurthy S, Kleman AM, LE Landree, Aja S. AMPK in the brain: its roles in energy balance and neuroprotection. J Neurochem 2009;109:17–23. [CrossRef]
10. Kulkarni SK, Dhir A. Berberine: a plant alkaloid with therapeutic potential for central nervous system disorders Phytother Res 2010;24:317–324. [CrossRef]
11. Ahmed T, Gilani AUH, Abdollahi M, et al. Berberine and neurodegeneration: A review of literature. Pharmacol Rep 2015;67:970–979. [CrossRef]
12. Xie X, Chang X, Chen L, et al. Berberine ameliorates experimental diabetesinduced renalinflammation and fibronectin by inhibiting the activation of RhoA/ROCK signaling. Mol Cell Endocrinol 2013;381:56–65. [CrossRef]
13. Wang Y, Huang Y, Lam KS, et al. Berberine prevents hyperglycemia-induced endothelial injury and enhances vasodilatation via adenosine monophosphate-activated protein kinase and endothelial nitric oxide synthase. Cardiovasc Res 2009;82:484–492. [CrossRef]
14. Chen Z, Peng IC, Sun W, et al. AMP activated protein kinase functionally phosphorylates endothelial nitric oxide synthaseSer633. Circ Res 2009;104:496–505. [CrossRef]
15. Dudhani RV, Kyle M, Dedeo C, Riordan M, Deshaies EM. A low mortality rat model to assess delayed cerebral vasospasm after experimental subarachnoid hemorrhage. J Vis Exp 2013;71:e4157. [CrossRef]
16. Zhou XQ, Zeng XN, Kong H, Sun XL. Neuroprotective effects of berberine on stroke models in vitro and in vivo. Neurosci Lett 2008;447:31–6. [CrossRef]
17. Zuccarello M, Kassell NF, Sasaki T, Fujiwara S, Nakagomi T, Lehman RM. Barrier disruption in the major cerebral arteries after experimental subarachnoid hemorrhage in spontaneously hypertensive and normotensive rats. Neurosurgery 1987;21:515–22. [CrossRef]
18. Koźniewska E, Michalik R, Rafałowska J, et al. Mechanisms of vascular dysfunction after subarachnoid hemorrhage. J Physiol Pharmacol 2006;57:145–160. https://pubmed.ncbi.nlm.nih.gov/17244946/
19. Sasaki T, Kassell N, Zuccarello M, et al. Barrier disruption in the major cerebral arteries during the acute stage after experimental SAH. Neurosurgery 1986;19:177–184. [CrossRef]
20. Osuka K, Watanabe Y, Usuda N, Atsuzawa K, Yoshida J, Takayasu M. Modification of endothelial nitric oxide synthase through AMPK after experimental subarachnoid hemorrhage J Neurotrauma 2009;26:1157–1165. [CrossRef]
21. Jeon H, Ai J, Sabri M, et al. Neurological and neurobehavioral assessment of experimental subarachnoid hemorrhage. BMC Neurosci 2009;10:103. [CrossRef]
22. Chen S, Feng H, Sherchan P, et al. Controversies and evolving new mechanisms in subarachnoid hemorrhage. Prog Neurobiol 2014;115:64–91. [CrossRef]
23. Hanggi D, Steiger HJ. Nitric oxide in subarachnoid haemorrhage and its therapeutics implications. Acta Neurochir 2006;148:605–613. [CrossRef]
24. Sabri M, Lass E, Macdonald RL. Early brain injury: a common mechanism in subarachnoid hemorrhage and global cerebral ischemia. Stroke Res Treat 2013;2013:394036. [CrossRef]
25. Nagata D, Mogi M, Walsh K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. J Biol Chem 2003;278:31000–31006. [CrossRef]
26. Zhang L, Li Z, Feng D, et al. Involvement of Nox2 and Nox4 NADPH oxidases in early brain injury after subarachnoid hemorrhage. Free Radic Res 2017;51:316–328. [CrossRef]
27. Higashi M, Shimokawa H, Hattori T, et al. Long-term inhibition of Rho-kinase suppresses Angiotensin II-induced cardiovascular hypertrophy in rats: in vivo effect on endothelial NADPH oxidase system. Circulation research 2003;93:767–775. [CrossRef]
28. Sabri M, Ai J, Lass E, D'abbondanza J, Macdonald RL. Genetic elimination of eNOS reduces secondary complications of experimental subarachnoid hemorrhage. J Cereb Blood Flow Metab 2013;33:1008–1014. [CrossRef]
29. Yao L, Romero MJ, Toque HA, Yang G, Caldwell RB, Caldwell RW. The role of RhoA/Rho kinase pathway in endothelial dysfunction. J Cardiovasc Dis Res 2010;1:165–170. [CrossRef]
30. Ayer RE, Zhang JH. Oxidative stress in subarachnoid haemorrhage: significance in acute brain injury and vasospasm. Acta Neurochir Suppl 2008;104:33–41. [CrossRef]
31. Won SJ, Tang XN, Suh SW, Yenari MA, Swanson RA. Hyperglycemia promotes tissue plasminogen activator induced hemorrhage by increasing superoxide production. Ann Neurol 2011;70:583–590. [CrossRef]
32. Vallet P, Charnay Y, Steger K, et al. Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia. Neuroscience 2005;132:233–238. [CrossRef]
33. Daou BJ, Koduri S, Thompson BG, Chaudhary N, Pandey AS. Clinical and experimental aspects of aneurysmal subarachnoid hemorrhage. CNS Neurosci Ther 2019;25:1096–1112. [CrossRef]