**Abstract.** Clinical studies have indicated that early brain injury (EBI) following subarachnoid hemorrhage (SAH) is associated with fatal outcomes. Oxidative stress and brain edema are the characteristic pathological events in occurrence EBI following SAH. The present study aimed to examine the effect of 3,4-dihydroxyphenylethanol (DOPET) against SAH-induced EBI, and to demonstrate whether the effect is associated with its potent free radical scavenging property. SAH was induced in rats using an endovascular perforation technique, and 24 h later the rats displayed diminished neurological scores and brain edema. Furthermore, elevated malondialdehyde (an index of lipid peroxidation) and depleted levels of antioxidants were observed in the rat cerebral cortex tissue. Quantitative polymerase chain reaction analysis indicated the upregulated mRNA expression of the apoptotic markers caspase-3 and -9 in the cerebral cortex. Furthermore, the protein expression levels of the proinflammatory cytokines tumor necrosis factor-α, interleukin (IL)-1β and IL-6 were significantly upregulated in SAH-induced rats. By contrast, treatment with DOPET significantly attenuated EBI by reducing brain edema, elevation of antioxidant status, inhibition of apoptosis and inflammation. In this context, DOPET may be a potent agent in the treatment of EBI following SAH, as a result of its free radical scavenging capacity.

**Introduction**

Globally, subarachnoid hemorrhage (SAH) is a prominent pathological occurrence which is involved in the etiology of 5-7% of all strokes cases that involve high mortality and functional loss (1). Despite advances in medical treatment and diagnosis, the mortality and morbidity rates of SAH have not been decreased significantly (2). Furthermore, the outcome of treatment in SAH patients remains poor, with a mortality rate of >50% and high morbidity among the survivors (3-5). Due to its complex pathology, researchers have deepened their interest in understanding the mechanisms underlying SAH at molecular level (6).

In the event of SAH, two clinical scenarios have been addressed primarily; vasospasm and early brain injury (EBI) (7). Arterial narrowing during SAH elicits fatal complications such as cerebral ischemia, and hence targeting the vasospasm has been a key target in the treatment of SAH among neurosurgeons in the past years (4). However, little success has been achieved in improving outcome following SAH (8,9). Additionally, accumulating studies have indicated that the administration of clazosentan in SAH patients does not improve patient outcome, despite reducing vasconstriction (8,10). Therefore, studies have investigated the involvement of cardinal factors such as ischemia, disruption of the blood brain barrier (BBB), inflammatory reactions and cortical spreading depression in the early stages following SAH (11,12).

Previous studies have suggested that the EBI period (24-72 h) following SAH elicits a series of events that may lead to poor prognosis (13,14). Furthermore, prior reports indicate that oxidative stress and brain edema are involved in EBI after SAH (15,16). Furthermore, reactive oxygen species (ROS) and reactive nitrogen species have been implicated in the occurrence of brain injury after SAH (17).

During EBI, brain edema occurs due to the disruption of the BBB (18,19) more than as a consequence of vasospasm (16). Thus, in clinical diagnosis, global edema has been proposed as a sole independent risk factor for fatal complications after SAH (16). Furthermore, noxious oxidative assault has been closely associated with brain edema (19). Therapeutic approaches have focussed on inhibiting ROS-induced apoptosis and inflammation as reasonable choices for the treatment of brain injury (20). Among an array of therapeutic interventions, a potential approach to boost or combat endogenous defense against oxidative stress is through dietary or pharmacological intake of antioxidants (21).

3,4-Dihydroxyphenylethanol (DOPET) is a phenol extracted from olive oil and grape juice, and is an endogenous metabolite of dopamine (22). DOPET has a good
safety margin (23), and has been suggested to exert neuroprotective (24,25), cardioprotective (26), uroprotective (27), renoprotective (28), hepatoprotective (29), anti-diabetic and antiobesity (30), anti-osteoporotic (31), anti-inflammatory (32), anti-atherosclerotic (33), anticarcinogenic (34) and anti-virus effects (35) in animal studies. Notably, earlier reports indicate the neuroprotective potential of DOPET in rats and in vitro (36-38). On the basis of these preliminary findings, we investigated whether DOPET may be an effective molecule in the in the mitigation of SAH in a rat model.

Materials and methods

Animals. A total of 21 male Sprague-Dawley rats (weight, 170-200 g; age, 9 weeks) were obtained from the animal facility of Tongcheng People’s Hospital (Xianning, China). The animals were maintained under standard laboratory conditions of relative humidity (55±5%), temperature (25±2°C) and light (12-h light/dark). The rats were fed standard diet pellets and water was provided ad libitum.

Animal grouping. Sprague-Dawley rats were divided into three groups (n=7 per group): Sham-operated rats (sham group); SAH rats treated with saline (SAH group); and SAH rats treated with DOPET (10 mg/kg) orally (SAH + DOPET group).

Administration of DOPET. DOPET was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline at a concentration of 3%. In the SAH + DOPET rats, DOPET (10 mg/kg) was injected intraperitoneally at 5 min and 6 h after SAH induction. In the SAH group, the rats underwent SAH-induction and were treated with an equal volume of 0.9% saline. No treatment was applied in the sham-operated animals.

Induction of SAH. SAH in rats was induced using an endovascular perforation technique, as described previously (39). Briefly, in anesthetized rats (5% isoflurane; Sigma-Aldrich and a sulfhydryl compound. Through spectrophotometry, the method of SOD determination involves generation of superoxide radical by photoreduction of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride at 543 nm. One unit of SOD activity was defined as the amount of enzyme capable of inhibiting 50% of nitrite formation under assay conditions. All standards and samples were run in duplicate. Tissue protein concentrations were determined using a BCA Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription quantitative polymerase chain reaction. Total RNA was extracted from cerebral cortex tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to manufacturer’s instructions. A total of 0.1 µl RNA was reverse transcribed using Moloney murine leukemia virus RT (Thermo Fisher Scientific, Inc.) in a 30 µl reaction mixture. The resultant cDNA (20 ng) was amplified using an iCycler IQ real-time detection system (Bio-Rad Laboratories, Inc) using IQ Supermix with 0.5X SYBR-Green (Bio-Rad Laboratories, Inc.). β-actin served as an endogenous control. Rat-specific primers for caspase-3 and caspase-9 were synthesized by Shanghai Shine Gene Molecular Biotech, Inc. (Shanghai, China), and the sequences were as follows: Caspase-3, forward 5'-GGTATTGGACAGACAGTGG-3' and reverse 5'-CAT GGGATCTGTGTTCTTTCGC-3'; caspase-9, forward 5'-ACA
AGGCCCTCGACAGTG-3' and reverse 5'-GTACCAGGAACGCCCTTCT-3'; and β-actin, forward 5'-ATCTGGGAC CACACCTTCT-3' and reverse 5'-AGCCAGGTCCACACGC CA-3'. Thermocycling conditions were as follows: Initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at -95°C for 15 sec, annealing at -58°C for 45 sec and extension at -60°C for 30-45 sec, with final extension at 72°C for 5 min. mRNA expression levels were normalized to the β-actin internal reference gene and the relative expression levels were calculated using the 2^[-ΔΔCt] method (42) and CFX Manager software (Bio-Rad Laboratories, Inc.).

Western blot analysis. Western blotting was performed as described previously (43). Briefly, the left basal cortical sample facing the blood clot was weighed, homogenized, and centrifuged at 1,000 x g for 10 min at 4°C. The resulting supernatants were further centrifuged. Samples were transferred to sterile tubes containing cold TCAEB acetone [acetone containing 10% (w/v) TCA and 0.07% mercaptoethanol], and the proteins were precipitated for 1 h at -20°C, followed by centrifugation at 18,900 x g for 15 min at 4°C. The supernatant was decanted, and the pellet was washed twice with chilled wash buffer (acetone containing 0.07% mercaptoethanol, 2 mM EDTA and EDTA-free proteinase inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany), followed by the removal of the acetone. The pellet was subsequently solubilized in LB-TT [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 18 mM Tris-HCl (pH 8.0), 14 mM trizma base, EDTA-free proteinase inhibitor cocktail, 0.2% (v/v) Triton X-100 (R), containing 50 mM dithiothreitol]. The protein content was measured using a DC protein assay kit (Bio-Rad Laboratories, Inc.) prior to electrophoresis. An equal quantity of protein (60 μg) from each sample was resuspended in loading buffer (Bio-Rad Laboratories, Inc.), denatured at 95°C for 5 min, separated by 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membranes (both Bio-Rad Laboratories, Inc.). The membranes were blocked with non-fat dry milk buffer for 2 h and incubated overnight at 4°C with primary antibodies against interleukin (IL)-1β (cat. no. sc-7884; 1:500), IL-6 (cat. no. sc-13026; 1:800), tumor necrosis factor (TNF-α) (cat. no. sc-1351; 1:800) and β-actin (cat. no. sc-47778; 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membranes were processed with horseradish peroxidase-conjugated chicken anti-rabbit IgG secondary antibodies (1:500; Santa Cruz Biotechnology, Inc.) at room temperature for 3 h.

Statistical analysis. Data are presented as the mean ± standard error of the mean. SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. All data were subjected to one-way analysis of variance followed by the Tukey test for multiple comparisons. P<0.05 was considered to be statistically significant.

Results

Effect of SAH and DOPET on neurological score. The neurological score was significantly (P<0.05) decreased in the SAH group compared to the sham group. After DOPET treatment, neurological deficits were reduced compared to that of the SAH (P<0.05) (Fig. 1).

Effect of SAH and DOPET on brain water content. As shown in Fig. 2, brain water content was significantly (P<0.05) elevated in SAH group compared to the sham group at 24 h after SAH. Brain edema was attenuated significantly (P<0.05) reduced in the SAH + DOPET group compared with the SAH group.

Effect of SAH and DOPET on lipid peroxidation in brain cortex homogenate. Lipid peroxidation in the cerebral cortex was quantified by measuring MDA levels (Fig. 3). The level of MDA in the brain of SAH rats was significantly higher compared with in the sham rat group (P<0.05). The increase in lipid peroxidation indicates an elevated oxidative stress in the brain of SAH rats, which was significantly decreased by treatment with DOPET compared to SAH rats (P<0.05).

Effect of SAH and DOPET on oxidative stress. The concentration of ROS is determined by the balance between the rate of production and the rate of clearance by various antioxidant compounds and enzymes. In the present study, post SAH there was a significant (P<0.05) decline in the level of antioxidants (GSH, SOD, and GPx) when compared to the sham rats. Treatment with DOPET significantly (P<0.05) increased the level of antioxidant in brain through its anti lipid peroxidative effect (Fig. 4).

Effect of SAH and DOPET on caspase-3 and caspase-9 mRNA expression. In the experimental SAH model, the caspase-3 and caspase-9 mRNA expression levels in the cerebral cortex were significantly increased (P<0.05) when compared with the sham-operated rats. However, therapeutic intervention with DOPET downregulated the mRNA levels of caspase-3 and caspase-9 when compared with the SAH rats, and thus attenuated the apoptosis (Fig. 5).

Effect of SAH and DOPET on protein expression of pro-inflammatory cytokines. Western blot analysis was used to evaluate the protein expression levels of TNF-α, IL-6 and IL-1β. Compared with the sham group, levels of the three inflammatory cytokines were significantly increased 24 h after SAH in the SAH group (P<0.05), whereas DOPET administration significantly reduced the levels of TNF-α, IL-6 and IL-1β compared with the SAH group (P<0.05). These results show that administration of DOPET downregulates the cortical expressions of pro-inflammatory cytokines 24 h after SAH (Fig. 6).

Discussion

Oxidative stress is a biological event which emerges from the potent cellular oxidizing ability of abundant ROS or free radicals (44,45). Following SAH, increased generation of oxidative stress occurs and prior results suggest that oxidative stress is a prime mediator of brain injury (15). During SAH, clot derived hemoglobin (Hb) triggers free radicals,
including O$_{2}^•$, H$_2$O$_2$ and ‘OH, which subsequently react. Auto-oxidation of Hb produces O$_{2}^•$ and dismutation of two O$_{2}^•$ forms H$_2$O$_2$, which is the source of highly reactive ‘OH in the reaction catalyzed by ferric ion (46). Amongst these oxidants, ‘OH is highly potent and attacks the nucleic acids, lipids and proteins to produce a marked cytotoxic effect (47,48). Thus, the generation of ‘OH free radicals from extravasated Hb (49), loss of mitochondrial integrity (50) and depletion of endogenous antioxidant system (51) have been elsewhere reported in experimental or human SAH.

Lipid peroxidation (LPO) is a noxious biological event induced by the free radicals such as ‘OH, ONOO$^-$ and H$_2$O$_2$ resulting in structural alterations of membranes and functional impairment of cellular components. MDA, the end product of LPO, attacks the polyunsaturated fatty acids of the cell membrane and thus serves as an effective marker of free radical damage (52). Similarly, in the present study, elevated MDA levels were observed in the cortex of SAH rats, which is in corroboration with a previous report (53). Treatment with DOPET significantly mitigated the elevated MDA level. The anti-lipid peroxidative effect of DOPET may be due to its lipophilic and hydrophilic nature (54). The phenolic group may imbed in the membrane, acting as a chain-breaking inhibitor of lipid peroxidation (55).

Furthermore, the downregulation of antioxidant defense system may be crucially involved in the pathology of SAH (56). In the antioxidant defense mechanism, the primary protection is performed by SOD against oxidative stress and LPO (15). In the oxidative stress cascade, the
Superoxide radical is initially generated and converted into H$_2$O$_2$ and molecular oxygen by catalase or GPx (57). Thus, vital organs and tissues are more prone to oxidative stress attack, which may be due to reduced antioxidant levels (58). The non-enzymic antioxidant reduced GSH, terminates the vicious cycle of ROS by reacting with the single oxygen and hydroxide radical and thus prevents tissue damage (59). In the present investigation, SAH rats displayed diminished glutathione, SOD, GPx and levels in cortex tissue. However, treatment with DOPET restored the altered antioxidant status to normal which may be due to the scavenging of free radicals and inhibition of LPO (60).

Delayed global edema has been displayed as an independent predictor of mortality (16). Furthermore, post SAH provoked cerebral edema may prelude elevated intracranial pressure (ICP) and brain herniation, leading to irreversible brain damage or mortality (61). Clinically, brain edema are underscored as cytotoxic or vasogenic edema (62). The characteristic features of cytotoxic edema include swelling with intracellular fluid accumulation which resembles astrocyte swelling (63). In cases of vasogenic edema, disruption of BBB occurs which may lead to the accumulation of fluid surrounding the cells (64). Furthermore, studies suggest that the altered expression levels of aquaporins, BBB disruption, clot derived substances, secondary noxious events like elevated ICP and hypertension are actively involved in the progression of brain edema after SAH, and hypertension are involved in the pathogenesis of brain edema (65,66). Turbulence in the BBB permeability is a key event during the brain injury after SAH (66). Furthermore, in SAH patients with vasogenic edema, a direct noxious effect after BBB rupture have been proved clinically, as well as in experimental studies (67).

Furthermore, the edema increases the brain volume and thus extends the elevated ICP after SAH (68). Consequently, there is an elevation in ICP, which further reduces cerebral blood flow, leading to increased ischemia (65). In the present study, it was found that the brain water content increased obviously after SAH and administration of DOPET abated brain edema significantly. Previous reports suggest that DOPET mitigates brain edema in ischemic rats by reducing of BBB permeability (69).

However, oxidative stress can induce changes of enzymes which are apoptosis-related, including p53, caspase-3 and caspase-9 (70). Caspase-9 is an essential protein involved in the breakdown of procaspase-3 to caspase-3 (71). During SAH, caspase-3 was overexpressed in the cortical neurons and the upregulation of caspase-3 led to the apoptosis of neural cells and brain edema (72). The present data showed that the expression of caspase-3 and caspase-9 increased significantly in the experimental SAH group, while these expression levels may be reversed by DOPET administration. These results suggest that DOPET could inhibit proapoptotic enzymes via its antioxidant activity and exertion of a neuroprotection effect.

In conclusion, DOPET treatment significantly attenuated the toxic manifestation of SAH by preserving BBB integrity, inhibition of lipid peroxidation and restoration of antioxidant levels. Furthermore, the mRNA expression levels of the apoptotic markers caspase-3 and caspase-9 and the protein expression of proinflammatory cytokines TNF-α, IL-6 and IL-1β were downregulated DOPET intervention. Further studies on DOPET are required to elucidate the
neuroprotective mechanism involved in its protective effect against SAH trauma.

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References


