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Research article

# Effects of low-dose unfractionated heparin on early brain injury after subarachnoid hemorrhage in mice

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ABSTRACT

*Background:* Sphingosine kinase (SphK) 1 has been reported as an important signaling node in anti-apoptotic signaling. Heparin is a pleiotropic drug that antagonizes many pathophysiological mechanisms. In this study, we evaluated if heparin prevents early brain injury (EBI) after subarachnoid hemorrhage (SAH) by anti-apoptotic mechanisms including SphK1.

*Methods:* SAH was induced by endovascular perforation in mice, which were randomly assigned to sham-operated (n = 23), SAH + vehicle (n = 36), SAH + 10U heparin pretreatment (n = 13), SAH + 30U heparin pretreatment (n = 15), SAH + 10U heparin posttreatment (n = 31), and SAH + 30U heparin posttreatment (n = 23). At 24 hours post-SAH, neurological scores, brain water content and Evans blue extravasation were evaluated. Also, the expression of SphK, phosphorylated Akt, and cleaved caspase-3 was determined by Western blotting, and cell death was examined by terminal deoxynucleotidyl transferase–mediated uridine 5'-triphosphate-biotin nick end-labeling staining.

*Results:* Low-dose heparin posttreatment improved neurobehavioral function, brain edema, blood-brain barrier disruption and cell death in the cortex, associated with an increase in SphK1 and phosphorylated Akt, and a decrease in cleaved caspase-3. High-dose heparin had a tendency for increased SAH severity, which obscured the neuroprotective effects by heparin.

*Conclusions:* Low-dose heparin posttreatment may decrease the development of post-SAH EBI through anti-apoptotic mechanisms including sphingosine-related pathway activation.

## 1. Introduction

Early brain injury (EBI), which occurs within 72 hours following cerebral aneurysm rupture, has been established as a factor for poor outcomes after subarachnoid hemorrhage (SAH) [5]. Apoptosis is involved in the pathogenesis of EBI after experimental SAH [1,5].

Thrombin is a multifunctional serine protease coagulation protein [18], and acts through protease-activated receptors (PARs), which are expressed on the surface of various cell types including endothelial, epithelial, neuronal and glial cells [9]. Thrombin has been implicated in blood-brain barrier (BBB) disruption, brain edema, inflammation and apoptosis [20]. However, it has been reported that activation of PAR1, the principle thrombin receptor, induces or inhibits apoptosis, depending on the dosage of thrombin in human endothelial cells [31].

Heparin, a glycosaminoglycan, prevents the coagulation process by inhibiting the action of thrombin and the formation of stable fibrin clots [12]. The two preferred routes of administration of unfractionated heparin are continuous intravenous infusion and subcutaneous injection (SC). However, the clinical use of heparin as a protective or an anti-inflammatory agent has been held back by the fear of bleeding.

Sphingosine 1 phosphate (S1P) is generated from sphingomyelin, an integral component of plasma membranes, by the sequential action of sphingomyelinase, ceramidase, and sphingosine kinase (SphK) [16]. Many factors can alter SphK activity and regulate the subsequent S1P levels [16]. S1P regulates diverse biological processes including cell survival after binding to S1P receptor-1 to 5 [8]. It has been reported that SphK1 activity reciprocally controls levels of both proapoptotic ceramide (SphK1 inhibition) and antiapoptotic S1P (SphK1 activation) [21].

The aim of this study was to test following 3 hypotheses: 1) 2 doses

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of unfractionated heparin can be used safely as either pre- or post-treatment at the SAH occurrence in mice; 2) heparin posttreatment attenuates post-SAH EBI through antiapoptosis in mice; and 3) the antiapoptotic effect of heparin involves a sphingosine-related pathway including SphK1 activation.

## 2. Materials and Methods

### 2.1. Experimental Design and Animal Groups

The animal and ethics review committee at Loma Linda University approved all protocols. One hundred forty-one 8-week-old male CD-1 mice (30-38 g; Charles River, Wilmington, MA) were used for the study.

Animals were randomly divided into 6 groups and evaluated at 24 hours after surgery: sham-operated (n = 23), SAH + vehicle (n = 36), SAH + 10U heparin pretreatment (n = 13), SAH + 30U heparin pretreatment (n = 15), SAH + 10U heparin posttreatment (n = 31), and SAH + 30U heparin posttreatment (n = 23).

### 2.2. Mouse SAH Model

SAH endovascular monofilament perforation model was produced as described previously [1]. Briefly, animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg). A sharpened 4-0 monofilament nylon suture was advanced through the internal carotid artery (ICA) to perforate the anterior cerebral artery. In the sham surgery, the filament was advanced 5 mm through the ICA without perforating the artery. Body temperature was kept constant (37.5 ± 0.5 °C) during the operation.

### 2.3. Drug Administration

It was reported that daily SCs of unfractionated porcine heparin of 20U per mouse (average body weight, 33 mg), which is comparable to 600U/kg/d and within the human therapeutic dose range, was given safely [23]. Based on this reference, at two hours before and after SAH induction, 10U and 30U heparin (McGuff company Inc., Santa Ana, CA) was subcutaneously administered.

### 2.4. Severity of SAH

The severity of SAH was blindly evaluated using the SAH grading scale with high-resolution photographs at the time of sacrifice [1]. The SAH grading system was as follows: the basal cistern was divided into six segments, and each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood clot in the segment; grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood clot with recognizable arteries; and grade 3, blood clot obliterating all arteries within the segment. The animals received a total score ranging from 0 to 18 after adding the scores from all six segments.

### 2.5. Mortality and Neurological Scores

The neurological score was blindly evaluated at 24 hours after SAH as previously described [1,2]. The evaluation consisted of six tests that can be scored 0 to 3 or 1 to 3. These six tests included: spontaneous activity; symmetry in the movement of all four limbs; forelimbs outstretching; climbing; side stroking; and response to vibrissae (whisker stimulation). Animals were given a score of 3 to 18 in 1-number steps (higher scores indicate greater function). Mortality was calculated at 24 hours after SAH.

### 2.6. Brain Water Content (BWC)

Brains were quickly removed and separated into the left and right cerebral hemispheres and weighed (wet weight) at 24 hours after surgery (n = 9 per group). Next, brain specimens were dried in an oven at 105 °C for 72 hours and weighed again (dry weight). The percentage of BWC was calculated as ([wet weight-dry weight]/wet weight) X100% [1].

### 2.7. BBB Disruption

At 24 hours after operation, we injected a 2% solution of Evans blue dye (4 mL/kg of body weight) intraperitoneally, and allowed it to circulate for 3 hours. Under deep anesthesia, mice (n = 6 per group) were sacrificed by intracardial perfusion with phosphate-buffered saline (PBS), and brains were removed and divided into the same regions as the BWC study. Brain specimens were weighed, homogenized in PBS, and centrifuged at 15,000 g for 30 minutes. Then, 0.5 mL of the resultant supernatant was added to an equal volume of 50% trichloroacetic acid. After overnight incubation and centrifugation at 15,000 g for 30 minutes at 4 °C, the supernatant was taken for spectrophotometric quantification of extravasated Evans blue dye at 610 nm as described previously [2].

### 2.8. Western Blotting

The left cerebral hemisphere (perforation side) at 24 hours after SAH was used (n = 5 per group). Western blotting was performed as previously described [1] using the following primary antibodies: anti-SphK 1 (1:250, Abgent, San Diego, CA), anti-phospho-Akt (Ser473) (1:1000, Cell Signaling Technology, Danvers, MA) and anti-cleaved caspase-3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

### 2.9. Terminal Deoxynucleotidyl Transferase-mediated Uridine 5'-triphosphate-biotin Nick End-labeling (TUNEL) Staining

Animals were euthanized 24 hours after surgery and brains were processed as previously described [1]. Ten-micron-thick coronal sections at the level of bregma-1 mm (caudally) were cut with a cryostat (LM3050S; Leica Microsystems, Bannockburn, Ill). TUNEL staining with an in situ cell death detection kit (Roche, Mannheim, Germany) was performed (n = 3 per group). TUNEL-positive cells were counted in three continuous fields of the left temporal basal cortex per case at × 400 magnification, and expressed as the mean number of TUNEL-positive cells/$mm^2$ with a fluorescence microscope [1].

### 2.10. Statistics

Neurological scores were expressed as median ± 25th to 75th percentiles and other data were expressed as mean ± SD. Neurological scores were analyzed using Kruskal–Wallis tests, followed by Tukey's multiple comparisons. Other statistical differences were analyzed using unpaired t tests and one-way analysis of variance (ANOVA) with Tukey–Kramer post hoc tests. Differences in mortality were tested using Fisher's exact tests. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Mortality and SAH Grade

The mortality rate was not significantly different among the vehicle, 10U/30U heparin pretreatment and 10U/30U heparin posttreatment groups of SAH mice (36.1%, 13 of 36 mice; 30.8%, 4 of 13 mice; 40.0%, 6 of 15 mice; 25.8%, 8 of 31 mice; and 39.1%, 9 of 23 mice, respectively). No sham-operated mice died. There was no significant difference in SAH grade between the vehicle and each treatment groups, although the SAH + 30U heparin pretreatment group had more severe SAH compared with the SAH + 10U heparin pretreatment group and

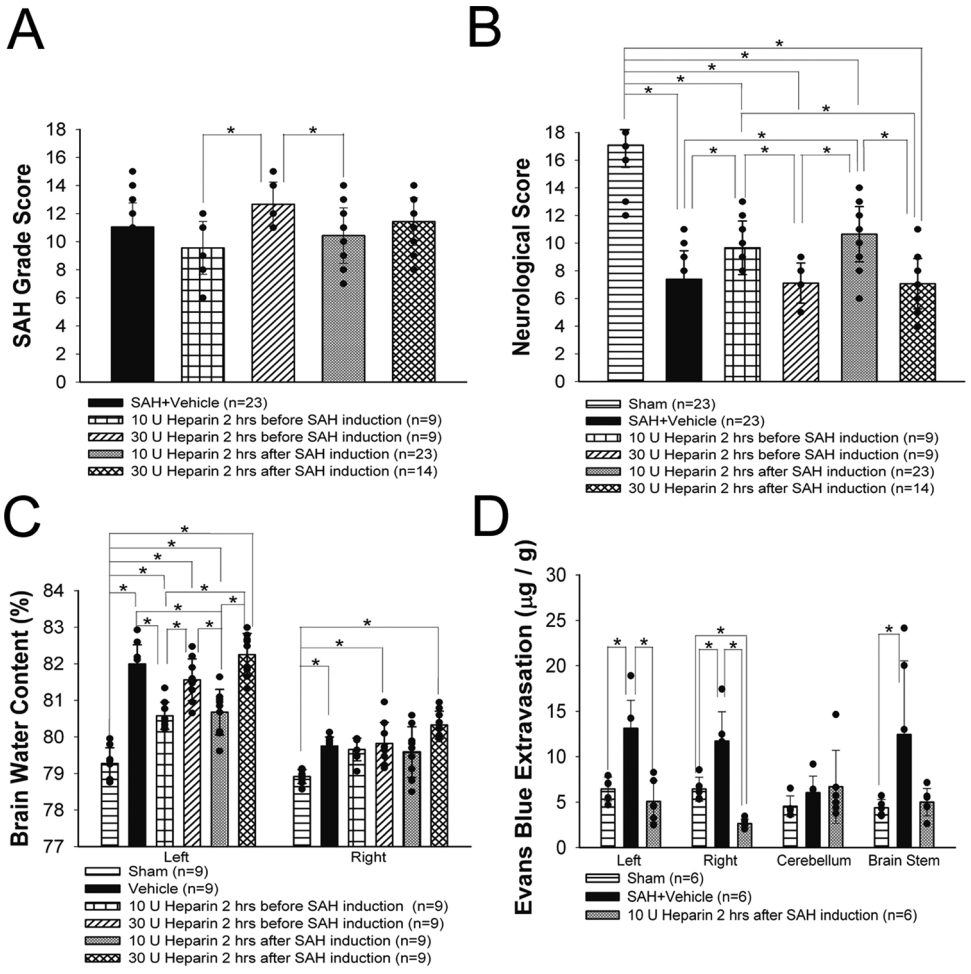


**Fig. 1.** SAH grade (**A**), neurological score (**B**), brain water content (**C**) and Evans blue dye extravasation (**D**) at 24 hours post-SAH. Values, median ± 25th to 75th percentiles (**A**, **B**) or mean ± SD (**C**, **D**); *P < 0.05, Kruskal-Wallis test (**A**, **B**) or ANOVA (**C**, **D**). Left, left cerebral hemisphere; Right, right cerebral hemisphere.

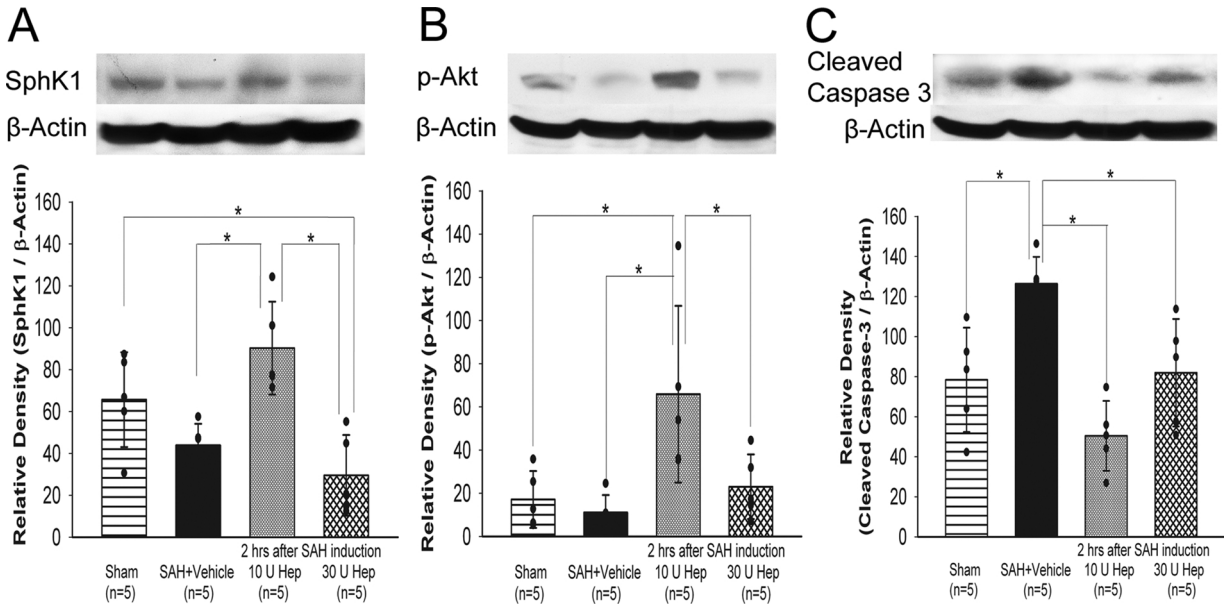


**Fig. 2.** Representative Western blots and quantitative analysis of SphK1 (**A**), p-Akt (**B**), and cleaved caspase-3 (**C**) in the left cerebral hemisphere at 24 hours after SAH. The protein band density values are calculated as a ratio of that of β-actin. Values are mean ± SD; *P < 0.05, ANOVA. Hep, heparin.

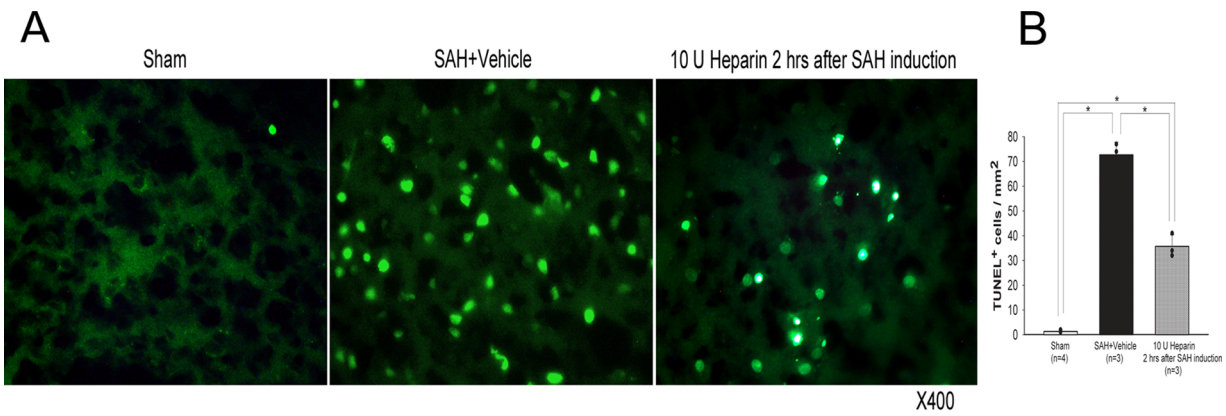


**Fig. 3.** TUNEL-positive cells (green) in the ipsilateral basal cortex at 24 hours after SAH. **A**, representative immunofluorescence images; **B**, quantitative analysis; values, mean ± SD; *P < 0.05, ANOVA.

the SAH + 10U heparin posttreatment group (P = 0.004, P = 0.019, respectively; Fig. 1A).

### 3.2. Neurological Score and BWC

Neurological score was significantly worse after SAH, and a significant improvement was observed only in the 10U heparin pre- and post-treatment groups compared with the vehicle group at 24 hours after SAH (Fig. 1B). BWC in the left hemisphere was significantly suppressed in the SAH + 10U heparin pre- and post-treatment groups, but the 30U heparin treatment was not protective (Fig. 1C). The 10U heparin posttreatment also inhibited post-SAH BBB disruption (Fig. 1D).

### 3.3. Effect of Posttreatment Low-dose Heparin on Sphingosine-related Antiapoptotic Pathway

Western blot analyses showed that SphK1 (Fig. 2A) and phosphorylated Akt (p-Akt) (Fig. 2B) were significantly increased in the SAH + 10U heparin posttreatment group compared with the SAH + vehicle group, associated with a significant decrease in cleaved caspase-3 expression (Fig. 2C). The SAH + 30U heparin posttreatment group had no effects on both SphK1 and p-Akt expressions, but somewhat decreased cleaved caspase-3 levels compared with the SAH + vehicle group.

Consistent with the Western blot results, TUNEL-positive cells in the left basal cortex were significantly increased after SAH, and significantly decreased by the 10U heparin posttreatment (Fig. 3).

## 4. Discussion

The present study showed that low-dose heparin posttreatment attenuated EBI at 24 hours after SAH, and that the protective effect was associated with decreased apoptosis. This antiapoptotic effect needed at least partly sphingosine-related pathway activation including SphK1.

Deleterious effects from SAH include disruption of the BBB, amplification of inflammatory infiltrates, and possibly disruption of cell-cell and cell-matrix interactions, which may trigger cell death. Apoptosis is a common feature of brain injury and contributes significantly to neuron and glial cell loss [6]. Apoptosis is believed to occur after acute injury such as EBI after SAH, cerebrovascular trauma or ischemia. Under such a situation, thrombin is produced and dramatically upregulated immediately at sites of cerebrovascular injury and may persist for days after injury [25]. At concentrations below 100 pM, the PAR1-dependent signaling activity of thrombin is protective, but at concentrations of higher than 100 pM, thrombin induces a pro-inflammatory response through the activation of PAR1 in endothelial cells [3] or thrombin-induced PAR1 activation engages in multiple signaling mechanisms in multiple cell types, leading to neuronal injury [10,11].

SphK is a conserved lipid kinase that catalyzes sphingosine into the form of S1P, a bioactive lipid or an important regulator of inter- and intracellular signaling [22]. The bulk of SphK activity is cell-associated, implying that S1P is formed in the cytosolic phase of the plasma membrane and is exported to the external leaflet of the plasma membrane so that it can be extracted by plasma chaperones such as high-density lipoprotein (HDL) and albumin [14,32]. S1P is abundant in plasma and more than 60% of the S1P in plasma is bound to lipoproteins [14,32]. Physiologically, lipoproteins may act as a protective mechanism to remove excessive S1P from plasma and prevent activation of S1P receptors [19]. S1P binds to S1P receptors with high affinity and launches typical G protein couple receptors-mediated signaling pathways [27]. S1P has been implicated in a number of agonist-driven cellular responses, including stimulation of cell proliferation, and inhibition of apoptosis [27]. S1P tissue level is low and kept under control through equilibrium between its synthesis mostly governed by SphK1 and its degradation by S1P lyase [26,28].

In 1954, Korn [15] established that the lipolytic activity of the lipoprotein lipase enzyme is heparin-dependent. The disappearance of lipoprotein lipase activity appears to parallel the circulating heparin level [15]. Endothelial lipase (EL) is a member of the triglyceride lipase family, which includes lipoprotein lipase and hepatic lipase. EL is principally a phospholipase, with nominal triglyceride lipase activity [13]. EL is synthesized principally by endothelial cells [30] and hydrolyzes HDL much more efficiently than other lipoproteins [17]. HDL particles represent the predominant S1P-carrier in plasma [7,24]. Free S1P is also protective at low concentrations [4], but proinflammatory and harmful at high concentrations [29].

Approximately one third of an administered dose of heparin binds to antithrombin III, and this fraction is responsible for most of the anticoagulant effect or anti-thrombin effect [12], possibly causing increased SphK1 and S1P, and therefore the antiapoptotic effects. The remaining two thirds have minimal anticoagulant activity at therapeutic concentrations, but at concentrations greater than those usually obtained clinically, possibly effected high amount of HDL hydrolysis and the resultant over-physiological levels of free S1P may antagonize heparin's protective effects. In addition, 30U heparin showed a tendency to increase SAH volume, which potentially causes more severe brain injury associated with a decrease in SphK1 expression. Thus, it is suggested that 10U heparin was protective, but 30U heparin lost the protective effects as this study demonstrated.

This study showed a new protective mechanism of heparin against post-SAH EBI, and the importance of dosages of heparin to exert protective effects in post-SAH EBI. Further studies would prove that this clinically available drug, heparin, is useful to treat post-SAH EBI.

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