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EVALUATION OF *INVITRO* THROMBOLYTIC, ANTI-INFLAMMATORY AND ISCHEMIC PROTECTIVE EFFECT OF ETHANOL AND CHLOROFORM FRACTIONS OF *TAXILLUS HEYNEANUS* CRUDE EXTRACT

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ABSTRACT
The purpose of study was to evaluate the *invitro* thrombolytic, *invitro* anti-inflammatory and ischemic protective effect of ethanol (ETH) and chloroform fractions (CTH) of *Taxillus heyneanus* whole plant crude extract.The thrombolytic activity of ETH, CTH and standard streptokinase by clot lysis method was found to be 33.32±0.81, 27.14±1.70 and 72.86±1.09 respectively. Both the fractions (ETH & CTH) of extract screened for *invitro* anti-inflammatory (human red blood cell membrane stabilization method) showed significant dose dependent anti-inflammatory activity with maximum protection of 71.59±1.13 and 59.36±1.00 when compared standard Diclofenac 90.18±1.49. For ischemic protective effect, rats were pretreated with different doses (200 & 400 mg/kg b.wt) of ETH and CTH for a month and ischemia was induced by Bilateral common carotid artery occlusion (BCCAO) for 30 mins followed by reperfusion for 45 mins along with ferric chloride induced thrombosis in one of the common carotid arteries. We measured infarct size,brain weight and brain water content. Pre-treatment with ETH and CTH reduced the brain weight brain edema and infarct size which may be attributed to the antioxidant,anti-inflammatory and thrombolytic activity of *Taxillus Heyneanus* extract fractions. From our findings, it is observed that high dose of ETH revealed remarkable ischemic protective activity. Further studies to observe *invivo* clot dissolving potential and to isolate active component(s) of extract fractions will be fruitful.

KEYWORDS: Anti-inflammatory, Ischemic protective, *Taxillus heyneanus*, Thrombolytic.

INTRODUCTION
Thrombo-embolic occlusion of cerebral blood vessel results in a pathological condition called the ischemic stroke which is leading cause of death and disability all over the world.[1] The blockage of extra or intracranial arteries holds up the nutrient and glucose supply to the respective brain region and initiates the cascade of ischemic events that ends with cellular dysfunction and death.[2]

Cerebral ischemia is not a simple condition to understand and treat. Years of research on the pathobiology of ischemic stroke reported the involvement of number of complex pathological processes such as hypoxia, energy failure, oxidative stress, calcium overload,excitotoxicity inflammation and apoptosis which are interrelated to each other.[3] Though multiple processes are involved in the pathobiology of stroke, immune system induced inflammation plays a critical role in its progression next to oxidative stress. Its mechanism is too complex involving both proinflammatory and anti-inflammatory mediators whose balance is disturbed by ischemia. Triggered by various stimuli the ischemic tissue secretes the inflammatory mediators such as cytokines and chemokines that upregulates adhesion molecules, recruites peripheral leukocytes and releases a variety of cytotoxic agents. All these subtances contribute to the disruption of blood brain barrier, hemodynamic deficits, brain edema and amplify the secondary brain damage.[4,5]

Though results of numerous animals studies indicate that inhibition of inflammatory responses could decrease brain injury and improve neurological outcome. But in clinical practice, the use of anti-inflammatory drug alone is of not much effective.[5] They might be effective when used along with thrombolytic therapy using recombinant tissue-type plasminogen activator (rtPA) within three hours of onset to clear the blockage of the occluded blood vessel and restore the blood flow to limit the brain injury.But it has limitations such as bleeding risk, narrow therapeutic window and ischemic reperfusion injury (IRI).[6]

As there is lack of effective neuroprotective drug till now, the focus of researchers is on the bioactive drugs with less side effects and multifactorial in action that targets different pathological process of ischemic stroke. Many phytomedicines obtained from plants afford neuroprotection via different mechanisms.[7] In that attempt, we have chosen the *Taxillus heyneanus* Danser for the present study.

Taxillus heyneanus Danser from Loranthaceae, a parasitic herb commonly called as badanika grows on shrubs and small trees of dry deciduous forests. Branches are densely covered with white shiny abietiform and stellate hairs. Tawny pubescent branchlets and nodes with prominently ribbed leaf scars are present. Leaves are alternate ovate leaves with attenuate base and crustaceous hairs on both surfaces are seen.[8] In the present study, we evaluated the *invitro* thrombolytic, *invitro* anti-inflammatory and ischemic protective effect of ethanol and chloroform fraction (ETH & CTH) of *Taxillus heyneanus* crude extract.

MATERIALS AND METHODS

Chemicals

All chemical agents used were of analytical grade were purchased from Sigma Chemicals Co. (St. Louis, USA) or Himedia, Mumbai.

Preparation and Fractionation of crude extract of plant material

Whole plant material was cut, washed, shade-dried for several days. Then pulverized into coarse powder and stored at room temperature (RT) for future use. The dried coarse powder (500g) of plant extract was macerated with absolute ethanol at room temperature for 10 days in a clean and sterilized glass container with frequent agitation. Later it was subjected to filtration. The filtrate obtained was concentrated on a water bath maintaining 40°C to dryness and designated as a crude extract of TH. The crude extract obtained subjected to fractionation by suspending in hydro alcoholic (7:3% v/v) solution and extracted successively with petroleum Ether and chloroform using separating funnel.[9] Dried plant extracts of different solvents were weighed and stored for further use. PET Ether used as the defatting agent and the amount of PET ether (PTH) fraction obtained is so less to evaluate. So in the present study, the Ethanol (ETH) and chloroform fractions (CTH) of crude extract were screened for *invitro* thrombolytic and antinflammatory activity and ischemic protective effect.

Phytochemical Analysis

The fractions of crude extract ETH,CTH and PTH were subjected to standard tests to detect the presence of the phytochemical constituents.[10]

Invitro thrombolytic activity

4 ml of blood was collected from each healthy human volunteer (n=3) who has not taken oral contraceptives or anticoagulants for last 10 days. Collected micro centrifuge tubes were weighed (W_1). To each properly labeled tube, 0.5 ml of freshly collected blood was added and allowed to clot by incubating at 37^0C for 45 mins. Serum was withdrawn completely from each tube without disturbing clot after its formation and weighed (W_2). Clot weight was calculated from the difference of above weights (W_2-W_1). To each tube 0.1ml of concentration (10 mg/ml) extract fractions (ETH & CTH), streptokinase (positive control) and normal saline (negative control) were added separately and incubated for 90 mins for clot lysis. After 90 mins, the liquid part was removed and clot remnants were weighed.[11] Then, percentage clot lysis was calculated from equation mentioned below.,

% Clot lysis = {weight of clot after lysis/weight of clot before lysis} ×100 ----- (1)

Invitro anti-inflammatory activity

The human red blood cells (HRBC) membrane stabilization anti-inflammation method was used in the present study.[12] Chloroform and ethanolic extracts fractions of *Taxillus heyneanus* were used for evaluation. First, blood was collected from a healthy human volunteer who has not taken any NSAIDS for 10-14 days before the experiment was mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride) and centrifuged at 2500 rpm for 5 min. The cell suspension remained after supernatant removal was washed with a normal saline solution (0.9% w/v NaCl). Then centrifuged again and again until the supernatant was clear and colorless. The packed cell volume was measured and 10% suspension was made. Various concentrations of extracts fractions were prepared (100, 250, 500 and 1000 µg/ml) using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were added and incubated at 37 ^{0}C for 30 min. After that, it was centrifuged at 3,000 rpm for 20 min. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. The standard drug was also taken in the same concentrations. Control absorbance was found from solutions omitting the extracts. The percentage of HRBC membrane stabilization or protection was calculated using the formula mentioned below,

Percentage protection=100− ((OD of drug treated sample/OD of control) ×100) ----- (2)

Experimental Animals

The experiments were carried out in albino Wistar rats of either sex weighing 180–200 g and were procured from Anurag Pharmacy College, Kodad, India. They were kept under standard conditions at 23-25^{0}C 12 hr light/dark cycle and fed with standard diet and water *adlibitum*. Before the experiment, the animals were acclimatized for 1 week under laboratory conditions. The study was conducted in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals) guidelines and approved by

the Institutional Animal Ethical Committee (Registration no -1712/PO/a/13/CPCSEA).

Drugs and Extract administration

The Ethanol and chloroform fractions of *Taxillus heyneanus* extract were prepared as a uniform suspension using 1% tween 80 for oral administration in experimental animals.[13]

Acute toxicity studies

Acute oral toxicity test of fractions of extract ETH & CTH was carried out as per Acute Toxic Class Method described in OECD 423. Before dose, the animals fasted overnight. The incidence of mortality was checked for first 24 hours and daily thereafter for 14 days. Two arbitrary doses of 200 mg/kg and 400 mg/kg were selected for the study, as the extract fractions were found safe up to 2000 mg/kg without any sign of toxicity or mortality.[14]

Experiment schedule for stroke protective activity

The animals were divided into six groups each containing 8 animals. The test group animals received different doses (200 and 400 mg/kg) of ETH and CTH orally once daily for 30 days whereas sham operated and control groups received vehicle (1% Tween 80). The treatment schedule is mentioned below.[15]

Group I (SHAM): Sham operated rats treated with 1% tween 80 without occlusion/reperfusion/ferric chloride application

Group II (ISCHEMIC CONTROL): Rats treated with 1% tween 80 and occlusion/reperfusion/ferric chloride application

Group III (ETH-1): Rats treated with low dose (200 mg/kg p.o) of ETH and occlusion/reperfusion/ferric chloride application

Group IV (ETH-2): Rats treated with high dose (400 mg/kg p.o) of ETH and occlusion/reperfusion/ferric chloride application

Group V (CTH-1): Rats treated with low dose (200 mg/kg p.o) of CTH and occlusion/reperfusion/ferric chloride application

Group VI (CTH-2): Rats treated with high dose (400 mg/kg p.o) of CTH and occlusion/reperfusion/ferric chloride application

Induction of stroke

After 30 days of treatment, stroke was induced by the combination of Global Occlusion model (BCCAO) and focal thrombotic model (ferric chloride induced arterial thrombosis) followed by reperfusion. Ventral midline incision on the neck area was done on pre anesthetized rats (ketamine 80 mg/kg i.p. and xylazine 10 mg/kg i.m.) to expose common carotid arteries (CCA).[16] Vagus nerve accompanying the CCA were carefully separated and stroke was induced by occluding both carotid arteries with vascular clips for 30 mins.[17] Then, in the process of reperfusion vascular clips were clamped and removed alternatively over a period of 45 mins.[18] Finally, the filter paper (1×1mm) saturated with 25% $FeCl_3$ was applied proximal to the surface one of the common carotid arteries for 15 minutes.[19] During the application, care must be taken such that it should not come in contact with surrounding tissue or other blood vessels. The temperature was maintained at 37 ± 0.5°C throughout the surgery. The animals were sutured and allowed to recover by housing them in individual cages. Sham operated rats received the same surgical incision without ischemic induction.

Determination of Brain weight and Brain Water content

After 7 days of thrombotic ischemic reperfusion, rat brains were quickly harvested by decapitation. Cerebellum, brain stem and olfactory bulb were removed and the brain tissues were weighed immediately to obtain wet weight (WW) then subsequently dried in an oven for 48 hours at 105°C, and weighed again to get dry weight.[20] The brain water content was calculated as follows

%Brain water content = [(wet weight − dry weight)/wet weight] × 100%. ------- (3)

Determination of infarct size

The brains of rats were quickly removed after decapitation and sliced into 2 mm-thick coronal sections and incubated in 2% TTC at 37°C for 30 min. Then the brain sections were fixed with 10% formalin solution. Brain tissue stained bright red area indicates the Nonischemic area and the unstained area or white area indicates ischemic tissue.[21]

RESULTS

Preliminary phytochemical screening

From this, we observed that both the ethanol (ETH) and chloroform (CTH) fraction of extracts gave a positive result with the Shinoda, Dragendroff, Gelatin, Fehling test and ferric chloride tests which indicated the presence of flavonoids alkaloids, tannins, carbohydrates and phenolic compounds in both the fractions whereas the PET ether fraction showed only the presence of steroids.the results were seen in table 1.

***Invitro* thrombolytic activity**

90 minutes incubation of streptokinase (positive control) with clots showed significant (P values < 0.0001) clot lysis of 72.86 ± 1.09% whereas distilled water (negative control) treated-clots showed only 3.34 ± 0.69% clot lysis which is very negligible. Ethanolic fraction of *T.heyneanus* showed the highly significant (P values < 0.0001) clot lysis activity (33.32 ± 0.81%) compared to chloroform extract (27.14±1.70). Results were shown in Table 2.

***Invitro* anti-inflammatory activity**

The HRBC membrane stabilization *invitro* anti-inflammatory activity method was used for our study because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization indicates the anti-inflammatory activity of the extract fractions. The

anti-inflammatory activity of different concentrations (100, 250, 500, 1000 µg/ml) of ETH, CTH, and Diclofenac (standard) were shown in Table 3 & Fig 1. Both the extract fractions ETH and CTH showed dose dependent protection. At highest concentration (1000 µg/ml) ETH, CTH, and standard showed percentage protection of 71.59±1.13, 59.36±1.00 and 90.18±1.49 respectively.

Effect of ETH and CTH on brain weight and brain water content (BWC)

Thrombotic ischemia reperfusion produced significantly ($P < 0.0001$) high brain water content (85.54 ± 1.44) in the ischemic control group compared to sham group (73.61±1.24). Both doses of chloroform extract and low dose of EDI reduced the water content slightly (81.50± 0.77, 80.88± 0.49 and 79.14±0.35) compared to ischemic control groups whereas high dose of ethanol extract fraction showed high significant ($P < 0.001$) decrease in water content (75.29± 0.35) which indicates its protective effect against stroke induced edema. The results of BWC represented in Figure 2. However, there was a significant decrease of brain weight observed in treated groups compared to control ischemic group Figure 3.

Effect of ETH and CTH on infarct size

Ischemic damage was determined by TTC staining. Compared with the ischemic control grouped ETH treated groups decreased the ischemic area or the unstained area in dose dependent manner which indicates better protection whereas CTH treated groups showed comparatively less ischemic protection than ETH which is represented in Figure 4.

Table1: Results of preliminary phytochemical screening of ethanol (ETH),chloroform(CTH) and PET ether (PTH)fractions of crude extract of TH.

Phytoconstituents	ETH	CTH	PTH
Flavonoids	++	+	-
Saponins	-	-	-
Steroids	-	-	++
Cardiac glycosides	-	-	-
Proteins	-	-	-
Carbohydrates	+	-	-
Tannins	++	+	-
Alkaloids	++	+	-
Phenols	++	++	-

++ high concentration; + low concentration, −absence

Table 2: *Invitro* Thrombolytic activity (% Clot lysis) of fractions of *Taxillus heyneanus* crude extract.

S.No	Drug/Extract fraction	% Clot lysis
1	Distilled water (Control)	3.34±0.69
2	Streptokinase(Standard)	72.86±1.09
3	ETH	33.32±0.81
4	CTH	27.14±1.70

Results represented in mean ± SD (n = 3).

Table 3: Effect of different doses of Ethanolic (ETH) and Chloroform (CTH) extract fractions of *Taxillus heyneanus* on stabilization of Human Red Blood Cell (HRBC) Membrane.

S.No	Concentration (µg/ml)	% Protection		
		ETH	CTH	Diclofenac
1	100	31.56±1.16	28.32±1.27	48.86±2.78
2	250	43.06±4.00	34.02±2.96	63.26±2.41
3	500	57.66±1.79	43.22±3.21	79.23±1.52
4	1000	71.59±1.13	59.36±1.00	90.18±1.49

Results represented in mean ± SD (n = 3)

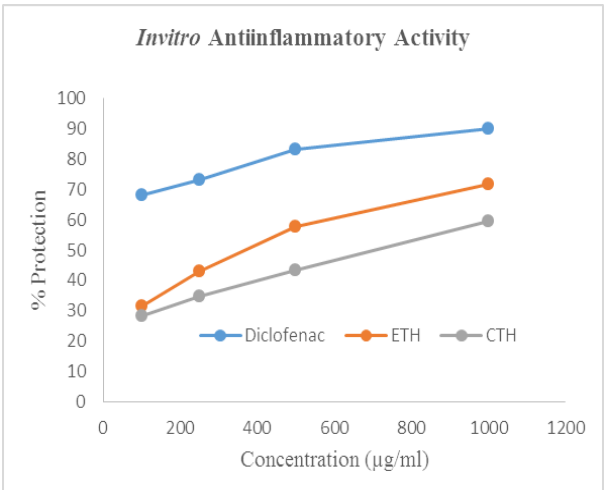

Figure 1: *Invitro* Anti-inflammatory activity of chloroform and ethanolic fractions of *Taxillus heyneanus* crude extract.

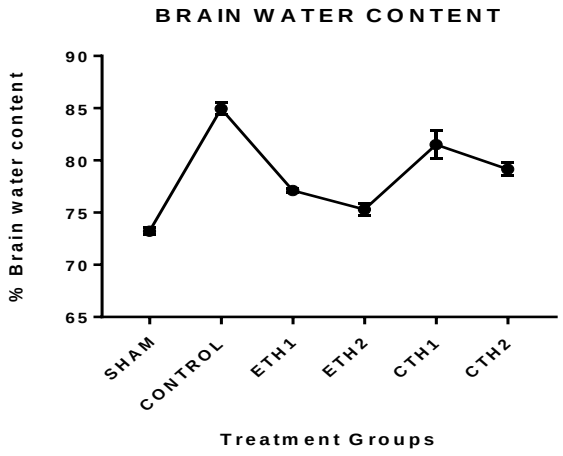

Figure 2: Effect of of different doses of Ethanolic (ETH) and Chloroform (CTH) fractions of *Taxillus heyneanus* crude extract on brain water content.

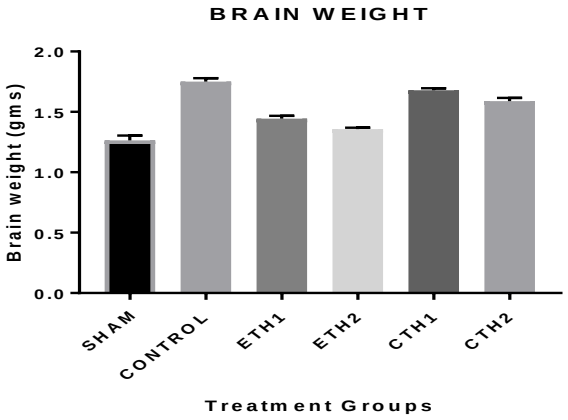

Figure 3: Effect of of different doses of Ethanolic (ETH) and Chloroform (CTH) fractions of *Taxillus heyneanus* crude extract on rat brain weight.

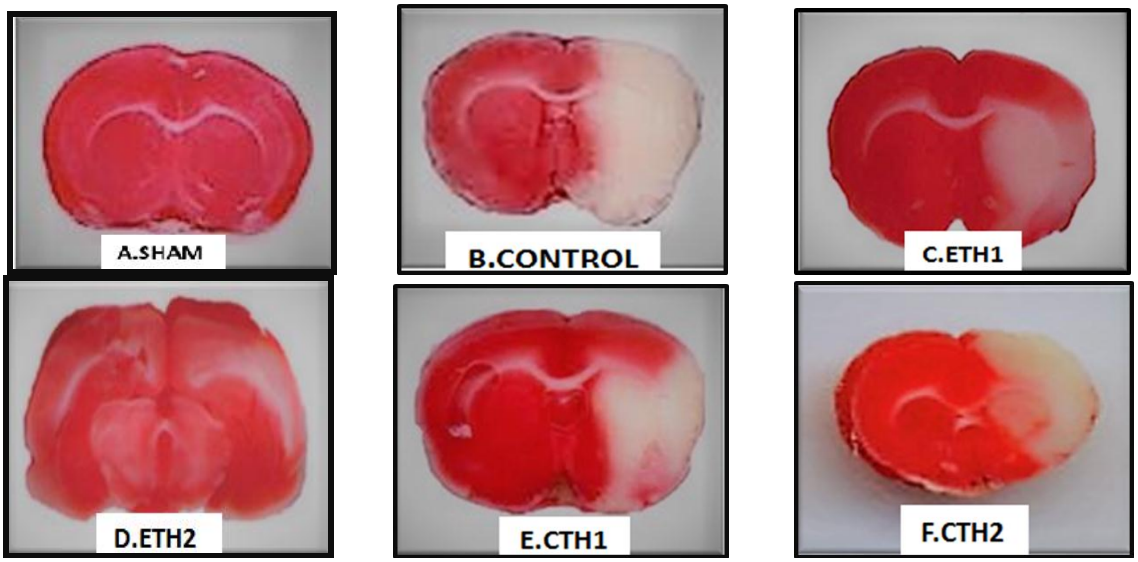

Figure 4: Representing TTC stained brain sections showing the ischemic core and penumbra at 7 days after Thrombotic Ischemic Reperfusion (TIR) brain injury of different treatment group rats. Pale or unstained area represents the ischemic area and red or stained area indicates normal healthy tissue.A.Rat receiving vehicle (sham) B.Control ischemic group C&D:Rats treated with low dose(200 mg/kg b.wt) & high dose (400 mg/kg b.wt) of ETH. E&F:rats treated with low dose(200 mg/kg b.wt) & high dose (400 mg/kg b.wt) of CTH

DISCUSSION

Factors such as genetic (inherited), acquired (autoimmune diseases) and environmental factors disturbs the hemodynamics of procagulant and anticoagulant systems resulting in the formation of thrombus,the insoluble product of coagulation cascade.[22] Formation of this, in cerebral blood vessel leads to impairment of blood flow to respective regions of brain resulting in pathological condition called ischemic stroke that involves many pathological processes including inflammation. Both thrombosis and inflammation are strongly related with each other. Inflammation produces local thrombosis and thrombosis amplify inflammation.[23] In the present study, we evaluated the *invitro* thrombolytic, *invitro* anti-inflammatory and ischemic protective effect of fractions of *Taxillus heyneanus* crude extract.

For ischemic protective effect evaluation, we have added the focal model of ferric chloride induced thrombosis to the Bilateral lateral common carotid artery occlusion method along with reperfusion to induce ischemia.To our surprise it produced severe ischemic damage than damage produced by each method individually which is evident from the increased brain water content and ischemic area in the control rat brains compared to sham group rats.

Brain edema, an inflammatory response associated with ischemic stroke. Ischemic tissue stimulate the release of proinflammatory and inflammatory mediators that disrupts the integrity of blood brain barrier,increase its permability and contribute to brain edema which was determined by calculating brain water content.[24] Pretreatment with ETH and CTH showed protective role

in thrombotic ischemic reperfusion injury by decreasing brain water content and thereby improving neurological function. This might be due to anti-inflammatory activity of fractions which was established in *invitro studies.*

The ischemic area was determined by TTC Staining technique which is the most reliable method and involves the principle of conversion of TTC to red stain formazan by dehydrogenase enzyme in normal live tissues where as absence of this enzyme in ischemic tissue leave it unstained.More the unstained or white area more is the ischemic damage.[25] In this study the combination of thrombosis,occlusion and reperfusion produced severe damage in control rat brains which is reduced by pretreatment with different doses of ETH and CTH that is indicated by less white or unstained area compared to control group. This might be due to the anti-inflammatory and thrombolytic action of the fractions of the crude extract TH reported *invitro.* Various studies reported that inflammatory processes, and thrombotic mechanisms are profoundly interconnected in predicting the outcome of cerebral ischemia. This multiple actions of fractions of TH may be due the presence of polyphenolic compounds such as flavonoids, alkaloids and tannins which exhibit different biological effects such as antioxidant, anti-inflammatory, antithromboic activity etc.[26] Further studies to isolate them and find its mechanism of action is necessary.

CONCLUSION

The ethanolic fractions (ETH) demonstrated comparitively high ischemicprotective effect than chloroform fraction (CTH) in this murine thrombotic ischemic reperfusion model. It suggest the potential relevance of ETH as a neuroprotective agent with multiple actions that may be beneficial for clinical applications in stroke. Further studies are needed to confirm the associations reported in this study by estimating various other inflammatory and thrombogenic biomarkers in rats with ischemic damage.

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