Reduction of brain injury using heparin to inhibit leukocyte accumulation in a rat model of transient focal cerebral ischemia. II. Dose–response effect and the therapeutic window

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The administration of massive doses of heparin has been demonstrated to reduce reperfusion injury. The authors have found that heparin’s antileukocyte adhesion property may play a more important role than its anticoagulant property in preventing ischemia and reperfusion injury. Although the administration of massive doses of heparin has been demonstrated to reduce brain injury after ischemia and reperfusion, the optimum dosage and timing for heparin administration remain unknown. The purpose of this study was to evaluate the dose–response effect and determine the time during which heparin must be administered to inhibit leukocyte accumulation, reduce infarct size, and improve neurological outcome in rats subjected to 1 hour of cerebral ischemia and 48 hours of reperfusion. Forty-nine animals were included in the study. The animals receiving commercial unfractionated heparin at a total dose of 2.67 to 4 mg/kg showed a significant inhibition of leukocyte accumulation, reduced infarct size, and lessened neurological dysfunction 48 hours after reperfusion (p < 0.05) when compared to untreated animals. The animals receiving unfractionated heparin within 3 hours after reperfusion also showed significantly better results than untreated animals. These data indicate that standard doses of heparin prevent reperfusion injury, and relatively late postischemic administration of heparin also is effective in brain protection. These findings may have therapeutic potential as an adjunct to thrombolytic therapy and possibly for other perfusion deficiencies with leukocyte–endothelial interaction. In view of these encouraging experimental findings, the clinical application of heparin administration after ischemia and reperfusion warrants serious consideration.

KEY WORDS • adhesion molecule • cerebral ischemia • heparin • leukocyte • rat
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ducted under the auspices of Research Animal Resources, a facility approved by the American Association for the Accreditation of Laboratory Animal Care.

**Experiment 1: Dose–Response Effect on Brain Protection.** Forty-two animals were used in Experiment 1. The animals were allocated to one of five groups: transient cerebral ischemia without treatment (Group I); treatment with intravenous administration of vehicle (saline) (Group II); treatment with intravenous administration of heparin (total 1.33 mg/kg; total 100 United States Pharmacopoeia (USP) U/kg) (Group III); treatment with intravenous administration of heparin (total 2.67 mg/kg; total 200 USP U/kg) (Group IV); or treatment with intravenous administration of heparin (total 4 mg/kg; total 300 USP U/kg) (Group V). The animals in Group I were the same as those used in Part I of this study. The animals in Groups II to V received two intravenously administered doses of 100 μl saline containing unfractionated heparin, one at the time of reperfusion and one 24 hours after reperfusion. Commercial unfractionated heparin from porcine intestinal mucosa was used in these studies.

**Experiment 2: Optimum Timing of the Administration of Heparin.** Seventeen animals were used in Experiment 2. The animals were allocated to one of two groups: heparin administration (total 4 mg/kg; total 300 USP U/kg) at 3 and 24 hours after reperfusion (Group VI) or at 6 and 24 hours after reperfusion (Group VII). The amount of heparin used in the study was that determined as the optimum dose in Experiment 1.

Seven animals from Experiment 1 and three animals from Experiment 2 were excluded from the study because they did not meet the selection criteria based on their neurological symptoms after induction of ischemia as described in Animal Preparation and Monitoring. Each group was composed of seven animals.

**Animal Preparation and Monitoring**

All procedures were performed with the animals completely anesthetized. Anesthesia was induced with intraperitoneal injection of a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg). The animals were then ventilated with a mixture of oxygen and air received via a face mask. The PaCO2 was maintained between 35 and 40 mm Hg. The rectal temperature was maintained between 37°C and 38°C with heating pads. The right femoral artery was cannulated for monitoring of arterial blood gas. A PE-10 catheter was introduced into the inferior vena cava via the right femoral vein for intravenous administration of vehicle or heparin.

Transient focal cerebral ischemia in the area perfused by the middle cerebral artery (MCA) was induced as described in detail in Part I. Briefly, the occluding device, a 4–0 nylon suture with a silicone-coated tip, was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the MCA. Reperfusion was accomplished by withdrawal of the suture. The animals underwent ischemia for 1 hour and reperfusion for 48 hours. After surgery, the rats were allowed free access to food and water. Neurological deficits characterized by left-sided hemiparesis and right-sided Horner’s syndrome were used as criteria for ischemic insult. Rats with convulsions or sustained consciousness disturbance, or without neurological deficits were excluded from the study. Most cases of sustained consciousness disturbance were due to subarachnoid hemorrhage caused by rupture of the intracranial internal carotid artery or MCA, and most cases of lack of neurological deficit were caused by unsuccessful MCA occlusion.

A neurological examination, as described by Zea Longa, et al., was performed in a blinded fashion by veterinary technicians and neurosurgeons 12, 24, and 48 hours after occlusion. A standard scoring scale was used: 0, normal; 1, failure to extend the left forepaw; 2, circling to the left; 3, falling to the left; and 4, no spontaneous walking and exhibition of a consciousness disturbance.

**Measurement of Infarction Size**

Four ischemic animals in each group were killed 48 hours after reperfusion. The size of infarction was measured as described in detail in Part I. Briefly, each brain was cut into 2-mm-thick coronal blocks, for a total of seven blocks per brain. The brain slices were incubated at 37°C for 30 minutes in 2% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC). The regions unstained by TTC, which reflect mitochondrial damage, were quantified as the infarcted areas. The surface of each slice was digitized, and the total and infarcted surface areas and volumes were calculated using commercially available reconstruction software. Measurement of the infarction’s size and macroscopic observation of brain sections were conducted in a blinded fashion.

**Myeloperoxidase Activity Assay**

The activity of myeloperoxidase, an enzyme localized in the azurophilic granules of leukocytes, is thought to be a quantitative index of polymorphonuclear leukocytes activity. For the biochemical determination of myeloperoxidase activity, three ischemic animals in each group were anesthetized and perfused transcardially with 150 ml of physiological saline (25°C at a pressure of 100 mm Hg) 48 hours after reperfusion. Brain samples of the ipsilateral (ischemic) and contralateral (normal) hemispheres were taken from the MCA area, immediately frozen in powdered dry ice, and stored at −80°C for later biochemical analysis. The method used to quantify myeloperoxidase activity from rat brain samples was described in detail in Part I of this study. Myeloperoxidase activity was assayed blindly as described, using a spectrophotometer. One unit of myeloperoxidase activity is defined as that which degrades 1 μmol of peroxide per minute at 25°C.

**Statistical Analysis**

All values are expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed on data derived from myeloperoxidase activity, size of infarction, and peripheral leukocyte counts. A two-way ANOVA with Tukey multiple comparisons was performed to compare the neurological outcome. A chi-square test was performed to analyze the incidence of hemorrhagic infarction. Differences were considered significant if p < 0.05.

**Sources of Supplies and Equipment**

Unfractionated heparin was obtained from Elkins-Sinn, Cherry Hill, NJ. Reconstruction software (version PC3D) was obtained from Jandel Corp., Corte Madera, CA.
Fig. 1. Experiment 1. Upper: Bar graph showing myeloperoxidase (MPO) activity. The MPO activity in the ischemic hemisphere of Groups IV and V shows a significant decrease when compared to Groups I and II (p < 0.05; asterisks). Center: Bar graph showing mean infarct volume expressed as a percentage of the total hemisphere for each study group. Vertical bars indicate standard deviation. Groups IV and V show a significant decrease in the size of infarction when compared to Groups I and II (p < 0.05; asterisks). Lower: Graph showing the clinical outcome of the animals in each study group. See Materials and Methods for definition of grades. The neurological grades of Groups IV and V are significantly better than those of Groups I and II (p < 0.05) at 24 and 48 hours after reperfusion (asterisks). MCA = middle cerebral artery.

Fig. 2. Experiment 2. Upper: Bar graph showing myeloperoxidase (MPO) activity. The MPO activity in the ischemic hemisphere of Groups V and VI shows a significant decrease when compared to Groups I and VII (p < 0.05; asterisks). Center: Bar graph showing mean infarct volume expressed as a percentage of the total hemisphere for each study group. Vertical bars indicate standard deviation. Groups V and VI show a significant decrease in the size of infarction when compared to Groups I and VII (p < 0.05; asterisks). Lower: Graph showing the clinical outcome of the animals in each study group. See Materials and Methods for definition of grades. The neurological grades of Groups V and VI are significantly better than those of Groups I and VII (p < 0.05) at 24 and 48 hours after reperfusion (asterisks). MCA = middle cerebral artery.
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4.74%; Group II, 44.93% ± 7.71%; Group III, 39.15% ± 3.43%; Group IV, 26.43% ± 6.66%; and Group V, 18.54% ± 4.73%. Groups IV and V showed a significant decrease in the size of infarction when compared to Groups I and II (p < 0.05). Group V showed a trend toward a smaller sized infarction than Group IV.

The incidence of hemorrhagic transformation in each group was as follows: Group I, 25%; Group II, 0%; Group III, 25%; Group IV, 0%; and Group V, 25%. There was no significant difference in the incidence of hemorrhagic transformation between groups. All hemorrhagic transformations observed in the basal ganglia were petechial.

Neurological Assessment. The neurological grades of the animals are summarized in Fig. 1 lower. The neurological grades of Groups IV and V were significantly better than those of Groups I and II (p < 0.05) at 24 and 48 hours after reperfusion. Group V showed a trend toward a better neurological outcome than Group VI 48 hours after reperfusion.

In summary, Groups IV and V showed a significant reduction in leukocyte accumulation, size of infarction, and neurological dysfunction after ischemia and reperfusion when compared to Groups I and II. Group V (4 mg/kg) showed a trend toward less myeloperoxidase activity and a smaller infarction size when compared to Group IV. Therefore, the total dose of 4 mg/kg of heparin was determined to be the optimum dosage, and this was used in Experiment 2.

Experiment 2: Optimum Timing of Heparin Administration

The animals in Group V in Experiment 1 received the same amount of heparin as those in Groups VI and VII at the time of reperfusion and 24 hours after reperfusion. Results from Experiment 2 are summarized in Fig. 2 with the data from Groups I (control) and V in Experiment 1.

Myeloperoxidase Activity. The mean myeloperoxidase activity in the ischemic hemisphere (Fig. 2 upper) was 0.11 ± 0.04 in Group VI (treated at 3 and 24 hours after reperfusion) and 0.19 ± 0.05 in Group VII (treated at 6 and 24 hours after reperfusion). Groups V and VI showed a significant decrease in the myeloperoxidase activity in the lesion when compared to Groups I and VII (p < 0.05). The mean myeloperoxidase activity in the contralateral hemisphere was 0.03 ± 0.04 in Group VI and 0.04 ± 0.02 in Group VII. There were no significant differences between groups in contralateral hemispheric myeloperoxidase activities.

Infarction Volume. The mean size of infarction (Fig. 2 center) was 23.59% ± 5.88% in Group VI and 39.73% ± 3.80% in Group VII. Groups V and VI showed a significant decrease in the size of infarction when compared to Group I (p < 0.05). There was no significant difference in the size of infarction between untreated animals and animals treated at 6 hours after reperfusion.

Neurological Assessment. The neurological grades of the animals are summarized in Fig. 2 lower. The neurological grades of Groups V and VI were significantly better than those of Group I (p < 0.05) at 24 and 48 hours after occlusion. There was no significant difference in neurological outcome between untreated animals and animals treated 6 hours after reperfusion.

Discussion

The current study demonstrates that intravenous administration of heparin at a total dose of 2.67 to 4 mg/kg clearly inhibits leukocyte accumulation in the ischemic tissue, reduces the size of infarction, and improves neurological outcome.

The dosage of heparin necessary to prevent brain injury after reperfusion was previously thought to be quite high.8,16–18 Smith, et al.,16 argued that amounts less than 5 mg/kg were not effective for brain protection after reperfusion in monkeys. However, in their study, they did not test the effect of smaller amounts of heparin for brain protection. The current study clearly demonstrates that intravenous administration of heparin at a total dose of 2.67 to 4 mg/kg prevents brain injury after ischemia and reperfusion. The animals in Groups IV and V received two doses of heparin, one at the time of reperfusion and one at 24 hours after reperfusion, each at a dose of 1.3 to 2 mg/kg per administration. The usual clinical dosage necessary to achieve routine anticoagulation is approximately 100 to 150 mg, administered every 6 to 8 hours; this is approximately 1.5 to 2 mg/kg for a person weighing 70 kg. Therefore, these data indicate that the standard doses of heparin can prevent brain injury after ischemia and reperfusion. We did not test the efficacy of the administration of massive doses of heparin because Group V (total 4 mg/kg) showed no significant difference in myeloperoxidase activity between the ischemic and normal hemispheres. The protective effect of heparin via the antileukocyte adhesion property was thought to be maximum at the dose of 4 mg/kg, and the risk of hemorrhage after administration of heparin at this dose level would be smaller than that from a massive dose. In Experiment 1, the incidence of hemorrhagic transformation in animals treated at the dosage level of 4 mg/kg was the same as that of untreated animals and there was no significant difference between groups.

It is advisable to use therapies for the inhibition of leukocyte adhesion in treating brain injury after transient but not permanent ischemia.2,23 Temporary arterial occlusion is often necessary during intracranial aneurysm surgery. However, when more than 10 to 20 minutes of temporary clipping is required, irreversible deficits due to ischemic and/or reperfusion injury will occur.18 Intravenous administration of heparin after a longer period of temporary arterial occlusion would be beneficial to prevent reperfusion injury. In addition, a recent study demonstrated that leukocyte inhibition using monoclonal antibodies against cell adhesion molecules enhanced the efficacy of thrombolytic therapy using tissue plasminogen activator.7 In contrast, Carter, et al.,3 reported that the administration of heparin with or in place of tissue plasminogen activator did not result in a significant decrease in the size of infarction in a rabbit model of embolic stroke. If heparin prevents reperfusion injury by blocking the function of leukocyte adhesion molecules, it should be of benefit when used in conjunction with thrombolytic therapy. In that study, the investigators induced 1 or 2 hours of cerebral ischemia and killed the animals only 5 hours after treatment. However, intercellular adhesion molecule-1, one of the leukocyte adhesion molecules, is present at low levels under normal conditions and is dra-
matically upregulated by cytokines; upregulation peaks 4 hours after stimulation and persists for several days.\(^2\) Although the efficacy of thrombolytic therapy itself may be estimated within a short period after ischemia, reperfusion injury occurs over a period of at least several days. Therefore, a longer period of observation would be necessary to estimate the efficacy of therapies designed to inhibit leukocyte adhesion. Moreover, ischemic tissue can be rescued by two therapeutic strategies: establishing reperfusion such as in thrombolytic therapy and hemodilution and the prevention of delayed injury by cytoprotective agents.\(^{15,19}\) Recent investigations suggest that leukocytes play an important role in the development of reperfusion injury by mechanical obstruction of vessels (plugging) and by biochemical mechanisms such as release of free radicals.\(^2,13\) Antileukocyte procedures may influence the reperfusion window by preventing leukocyte plugging and the cytoprotective window by reducing chemical mediators.

The current study indicates that heparin is effective in reducing the size of infarction when administered within 3 hours after reperfusion (4 hours after the onset of ischemia). These findings have positive implications for application of this form of therapeutic intervention in the clinical environment, which often demands a delayed intervention.

**Conclusions**

In conclusion, these studies clearly demonstrate the effectiveness of heparin in brain protection following ischemia and reperfusion injury. Heparin is easily administered, and has been shown to be safe in surgical situations. In view of these encouraging experimental findings, the clinical application of heparin administration after ischemia and reperfusion deserves serious consideration.

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**References**


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