Fibrinogen, Blood Viscosity, and Cerebral Ischemia

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SUMMARY This study examines the effect of fibrinogen and consequent blood viscosity reduction on cerebral blood flow and cellular injury following severe cerebral ischemia for 30 minutes in 78 Wistar rats. In half of these rats 10 to 15 cc’s of blood was removed and replaced with a mixture of 5% albumin and autologous red blood cells maintaining a constant hematocrit but resulting in a 30% decrease in fibrinogen and corresponding reduction in viscosity. Fibrinogen reduction resulted in a slight increase in baseline CBF and the elimination of post-ischemic hyperemia at 24 hours. Both study and control animals showed a similar decrease in CBF at 30 minutes and 2 hours. There was no significant difference in the severity of ischemic cellular change between the fibrinogen reduction group and controls, although there was a significant inverse relationship between the amount of viscosity change and severity of cellular injury within the treatment group. Fibrinogen reduction alone cannot significantly ameliorate ischemic injury in this model. Viscosity reduction therapy should include reduction of hematocrit and alteration of red cell deformability.

MANY POSSIBLE THERAPIES for acute cerebral ischemia alter blood viscosity, by hemodilution or phlebotomy, alteration of red blood cell deformability or reduction of fibrinogen concentration. The present study focuses on the effect of fibrinogen reduction alone on cerebral blood flow and cellular injury in an animal model of severe cerebral ischemia.

The viscosity of blood involves a complex relationship of red blood cells, fibrinogen, and the shearing forces within the vessel lumen (fig. 1). Several recent reviews have summarized how these rheologic factors may play a role in cerebral ischemia. Since a combination of blood viscosity, vascular tone, and intracranial pressure determine cerebral vascular resistance, lowering viscosity should increase CBF since CBF = perfusion pressure / cerebral resistance. Increased CBF and clinical improvement have been documented following viscosity reduction by phlebotomy and hemodilution. but these maneuvers effect viscosity primarily by decreasing hematocrit. Less attention has been focused on the effect of reducing fibrinogen, even though the concentration of this molecule is increased in cerebral ischemia.

Methods

Seventy-eight 300 gram male Wistar rats were fed ad lib. After anesthesia with 0.3 cc IM of a premixed cocktail of ketamine hydrochloride (one hundred milligrams per milliliter), Xylazine (20 milligrams per milliliter), and Acepromazine maleate (10 milligrams per milliliter) in a 3:3:1 ratio, all rats underwent bilateral vertebral artery cautery, and cannulation of the jugular vein and tail artery according to the method of Pulssinelli. After separating each common carotid artery from its sympathetic nerve trunk, loose ligatures were looped around the artery and the ligatures were delivered to the subcutaneous region. The skin was closed with metallic clips. Blood pressure was monitored throughout the procedure by connecting the tail artery to a Hewlett Packard transducer with strip chart recorder. (Hewlett Packard Co., Palo Alto California). Twenty-four hours after the initial surgery, the animals were exposed to ether for a few seconds and the skin clips removed. The common carotid arteries were

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delivered to the surface and, except in seven sham operated rats, both carotid arteries were clamped with aneurysm clips for thirty minutes resulting in a loss of righting reflex. Rats were included for further study only if loss of righting reflex persisted for the entire 30 minute period of carotid occlusion. In addition to blood pressure, rectal temperature was recorded by a thermistor probe, arterial blood gases were measured on a standard blood gas analyzer, hematocrit was determined using a capillary tube centrifuge, serum glucose was estimated by the dextrostix method, and osmolality was determined by freezing point depression. Fibrinogen was measured by a semi-automated technique. Plasma (0.5 ml) was mixed with a known quantity of thrombin, and fibrinogen concentration determined by comparing the plasma clotting time (inversely proportional to fibrinogen concentration) to a standard containing a known quantity of fibrinogen. Viscosity was measured using a Wells Brookfield microviscometer (Wells Brookfield, Inc., Stoughton, MA.), and a Rheometrics Fluids Rheometer (Rheometrics, Inc. Union, New Jersey) kindly supplied by Dr. Lawrence McIntire of Rice University. Seven rats had seizures during the post-ischemic period and were excluded from further analysis.

Immediately after release of the carotid clips, study rats underwent 10–15 cc phlebotomy via the tail artery while an equal volume of premixed autologous red blood cells plus five percent albumin was infused into the jugular vein. The exchange was carried out over 30 minutes in 4 cc increments and blood pressure was monitored intermittently throughout the procedure. There was only a slight rise in blood pressure in a small proportion of rats if the albumin was infused more rapidly than 1 cc per minute. The hematocrit of the premixed infusate was calculated to result in no change in the rats’ hematocrit from pre-exchanged values. Following the exchange repeat measurements of hematocrit, fibrinogen, viscosity and osmolality were performed. The infusate contained no glucose or dextrose, so that post-exchange glucose should not have differed from pre-exchange levels.

Neuropathological Studies

Twenty-six rats (14 study and 12 control) were subjected to neuropathological study. Three of these (2 study and 1 control) were sham operated and therefore not subjected to ischemia. Seventy-two hours after the ischemic insult or sham surgery, rats were infused with phosphate buffered 10% formalin, decapitated and their brains removed. In eight rats, a pigmented barium-gelatin mass was injected to demonstrate the microvascular supply to the regions being examined histologically. Mid-coronal slices three mm thick were embedded in paraffin, sectioned at five microns, and stained with hematoxylin and eosin. For microangiography, the paraffin blocks were placed directly on a glass emulsion plate and exposed in a Faxitron specimen X-ray unit.

Ischemic cell changes were quantitated in the hippocampus according to the percentage of cells with shrunken, eosinophilic cytoplasm and pyknotic nuclei. Although the involvement of other areas, such as corpus striatum and cortex, was observed in our animals and has been well described in the literature, the hippocampus was chosen for evaluation because the linear arrangement of the neurons facilitated quantitative observations. Care was taken to exclude “dark cell artifact” as described by Brierly et al. Severity of ischemic changes was graded in linear subdivisions of the dentate and Ammon’s horn regions as follows: 0–5% of cells involved; 6–25%; 26–50%; 50–75%; 76–100%. Observations were made by two independent observers (PO and DH) who were not aware of which treatment each rat had received. The results were plotted on diagrams of the sections to facilitate comparison. Rats were ranked by overall severity (proportion and linear extent) of ischemic damage. The
TABLE 1  Fibrinogen, Hematocrit, Osmolality, and Serum Glucose In Control And Exchanged Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pre-exchange</th>
<th>Immediately post-exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>366 ± 63 (n = 27)</td>
<td>359 ± 55 (n = 35)</td>
<td>251 ± 55 (n = 35)*</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>43 ± 10 (n = 30)</td>
<td>45 ± 7 (n = 36)</td>
<td>44 ± 10 (n = 36)</td>
</tr>
<tr>
<td>Osmolality</td>
<td>N.A.</td>
<td>317 ± 12 (n = 8)</td>
<td>313 ± 12 (n = 8)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>154 ± 54 (n = 5)</td>
<td>143 ± 40 (n = 8)</td>
<td>N.A.*</td>
</tr>
</tbody>
</table>

*Significant at p < .05.
†Since the infusate contained albumin + RBC's but no dextrose, pre-exchange and post-exchange glucose should not have differed.

Kruskal-Wallis test was performed and Spearman Rho was calculated for non-exchanged and exchanged groups.

CBF Studies

Forty-five rats (22 study and 23 control) had CBF studies using the 14C butanol technique. Ischemia and plasma exchange were carried out in the same fashion as in rats subjected to neuropathological study. Viscosity parameters, glucose, osmolality, and vital signs were monitored in the same fashion.

The rats were subdivided into three subgroups and CBF and arterial blood gases measured thirty minutes, two hours, and twenty-four hours after ischemia. There were five study and five control rats in the thirty minute and twenty-four hour subgroups, and ten study and eleven control rats in the two hour subgroup. An additional two study and two control rats were sham operated and CBF measured post-operatively. 5 uCi of 14C butanol was injected into the jugular vein during withdrawal of blood at a known rate through the tail artery. After ten seconds, the animal was decapitated.

The brain was rapidly removed and separated into right and left hemispheres, cerebellum and brain stem. Each brain segment was placed into pre-weighed sealed scintillation counting vials containing 1/2-2/3 cc protosol (New England Nuclear, Boston, MA) and 1 cc one hundred percent ethanol. The vials were then reweighed to obtain tissue weight. After heating in a water bath at 55° for twenty-four hours, 100-250 microliters of thirty percent hydrogen peroxide was added and the vials were resealed and reheated for thirty minutes at 50°. 10 cc of aquasol (New England Nuclear, Boston, MA) was then added and the vials shaken. 0.5 cc of 0.5N HCl was added to the vials which were then reagitated and placed into a scintillation counter (Searle Analytic, Inc. Mark II Liquid Scintillation System). The blood sample was handled in the same fashion except that the blood was mixed with 2 cc of a 1:2 mixture of protosol: ethanol, reweighed and heated for two hours at 50°. Five hundred microliters of thirty percent hydrogen peroxide was then added and the sample was subsequently handled just like the brain specimens. CBF was calculated according to the method of Van Uitert and Levy.

Results

1. Rheologic Parameters

Control and exchanged rats had comparable baseline hematocrit, fibrinogen, and glucose. Our technique of plasma exchange resulted in a 30% reduction of fibrinogen (p < .05) without affecting hematocrit or osmolality (table 1). However, because of the rapid hepatic synthesis of this molecule, fibrinogen returned to normal levels within twenty-four hours of a single exchange (fig. 2).

Fibrinogen reduction resulted in the predicted decrease in whole blood viscosity most apparent at low shear rates (fig. 3).

2. Cerebral Blood Flow

Arterial blood gases and mean arterial blood pressure were comparable in all groups of animals at the time of CBF measurement (table 2). In sham operated rats, fibrinogen reduction resulted in a significant (p = .05) increase in mean hemispheric CBF compared to controls (fig. 4), and there was a strong inverse statistical correlation between fibrinogen concentration and CBF (p < .01). Since our neuropathologic studies were confined to the cerebral hemispheres, we were most interested in hemispheric blood flow, though the response of CBF to viscosity reduction was the same in all regions of the brain measured including brainstem and cerebellum.

CBF in control rats 30 minutes, two hours, and 24 hours following ischemia was similar to values report-
ed by Pulsinelli in the same model, and Levy in gerbils. There was an initial severe depression of flow which returned toward normal within hours of reperfusion. By 24 hours, CBF was significantly higher than baseline (p = .05) indicating a period of delayed hyperemia.

There was no significant difference between the CBF of exchanged and control rats at 30 minutes and two hours following ischemic injury. At 24 hours, CBF was back to baseline in exchanged rats. Compared to controls, no hyperemia was observed. (p = .01)

3. Neuropathologic Studies

Exchanged and control rats demonstrated mild and equivalent amounts of weight loss (controls = 17 grams ± 4; exchanged = 17 grams ± 7) and listlessness over the 72 hours of observation post ischemia. In sham operated rats, no neuronal changes were observed in either the control or exchanged animals.

In rats subjected to 30 minutes of ischemia, predictable cell changes were found consisting of shrunken darkly staining neurons in both Ammon’s horn and dentate regions of the hippocampus 72 hours after ischemia (fig. 5). Microangiograms confirmed reperfusion of the microcirculation in these regions (fig. 6).

Ranking each hippocampus for severity of injury, we were unable to find a significant difference between the control and exchanged rats, even if comparisons were made between just the Ammon’s horn or dentate regions or by combining the scores of all regions. 42 percent of 48 regions examined in exchanged rats were free of severe injury compared to 53 percent of 36 regions examined in control animals (p > .05) indicat-
FIGURE 5A. Cellular changes in dentate (D) and Ammon's horn (A) 72 hours following a 30 minute period of ischemia in an exchanged rat. Normal cellular morphology is preserved in the dentate and portions of Ammon's horn (solid lines). A sharply demarcated area of ischemic cell change (broken line) is present in Ammon's horn.

FIGURE 5B. Higher power view of adjacent ischemic and relatively preserved areas in Ammon's horn. The ischemic neurons (arrows) have shrunken hyperchromatic nuclei, and pale shrunken cytoplasm.

...ing that fibrinogen reduction offered no protection from ischemia.

However, more protection did occur in those rats with greatest viscosity reduction. Within the exchanged group of rats, 54 percent of 28 regions examined in the sub-group with greatest reduction in viscosity were protected compared to 25 percent of 20 regions examined in the sub-group with least viscosity change (p < .05). Furthermore, there was a statistically significant inverse relationship between the amount of viscosity reduction achieved by removing fibrinogen, and the severity of ischemic injury (fig. 7).

Discussion

We used plasma exchange for fibrinogen reduction because we wanted to lower serum fibrinogen concentration rapidly and selectively. Clofibrate will lower fibrinogen over a two week period and urokinase and streptokinase' effect coagulation parameters in addition to viscosity, and may result in cerebral hemorrhage in clinical situations. Hossman and co-workers recently reported their findings using an cord, a viper venom extract having a relatively selective effect on fibrinogen, in human stroke patients.23

Although the animal model used in this study is one of global ischemia and not focal infarct, we feel it is appropriate for several reasons. Most importantly, predictable and measureable severe ischemic changes are produced. Since it is unlikely that any therapy will salvage infarcted tissue, it is reasonable to focus therapy on regions of severe ischemia. Recently, studies of patients with acute focal cerebral infarct using positron emission tomography have demonstrated ischemic regions around infarcts (i.e. ischemic penumbra) which may be threatened by further injury. These areas may demonstrate misery perfusion (i.e. low CBF but preserved metabolic function) and presumably may be salvaged by efforts to improve CBF, or become infarcted by failure to do so.

Reperfusion of the injured area of brain is another important advantage of this model since there would be...
little theoretic advantage to lowering blood viscosity if the blood was not recirculated to the threatened area. Using radiomicrographs we were able to demonstrate microvascular reperfusion in ischemic hippocampi.

Plasma exchange in this model proved to be a safe and effective method of fibrinogen and viscosity reduction, resulting in an elevation of baseline CBF in nonischemic animals. Only slight fluctuation of blood pressure was observed during the period of exchange and there was no effect on osmolality or hematocrit. No evidence of cellular injury was seen in exchange rats without ischemia, and no hemorrhage or other neuropathologic complications were found in exchanged post-ischemic rats.

One observable effect of fibrinogen reduction on post-ischemic CBF was the elimination of delayed hyperemia at 24 hours in exchanged rats. Many investigators feel that in reperfused ischemic tissue, hyperemia reflects greater tissue injury, representing maximum vaso-dilatation from the accumulation of acid metabolites or other vaso-active substances such as adenosine released following cellular injury. If these assumptions are correct, then our CBF data may reflect some beneficial effect of fibrinogen reduction.

Our neuropathologic data suggests that any beneficial effect of fibrinogen reduction is proportional to the amount of viscosity reduction at low shear rates. This consistency with a number of publications correlating degree of viscosity reduction by hemodilution with improvement in CBF and cerebral metabolism in animal stroke models. However, even the animals demonstrating greatest viscosity reduction in our study fared no better than controls. One explanation may be that the magnitude of viscosity reduction achieved by lowering fibrinogen alone is not sufficient to improve perfusion. Fibrinogen plays less of a role in the determination of viscosity than does packed red blood cell volume or red blood cell deformability in the microcirculation. Merrill calculated the relative contribution of fibrinogen and hematocrit in determining the yield shear strength (YSS) of blood which is the force required to initiate movement in a stationary blood column. YSS = 13.5 \(10^{-6}\) C_fib \(\text{Hct}^{-6}\), where C_fib = fibrinogen concentration and Hct = hematocrit. Fibrinogen is an order of magnitude less important in this relationship than is hematocrit. We have also confirmed in previous studies in humans and rats that fibrinogen correlates less strongly with viscosity and CBF than does hematocrit.

Nevertheless, we felt it was useful to determine the effect of lowering fibrinogen alone since fibrinogen concentration is affected by most current therapeutic efforts aimed at altering blood rheology such as hemodilution therapy or streptokinase. Our results indicate, however, that such therapy must be combined with a reduction of packed red cell volume or with an increase in red cell deformability to have any chance of success. This conclusion has recently been supported by Hossmann et al who found no significant clinical improvement following selective reduction of fibrinogen and viscosity by ancrod in patients with strokes.

Another explanation for the lack of benefit of fibrinogen reduction in this study lies in the animal model used and our study design. Pulsinelli has described hyperemia immediately after releasing the carotid clips in this model. Thus, cellular injury in this model may not be due to inadequate reperfusion at all, and therefore would not be ameliorated by viscosity reduction. Our data, therefore, indirectly supports the conclusion of Pulsinelli and co-workers that much of the cellular injury in this model is due to the cytotoxic effect of substances released at the time of ischemia. They demonstrated that despite reperfusion, there was worse ischemic cellular injury at 72 hours following ischemia than at 24 hours, and hypothesized that lactic acid accumulation produces ongoing cellular injury during that period. Others have postulated that calcium influx into the cell may produce irreversible cellular injury and that this might even be worsened during reperfusion.

Methodological limitations in our analytic techniques might have prevented the detection of some beneficial results. Perhaps regional measurements of cerebral blood flow and cellular metabolism by autoradiography would demonstrate a local effect of viscosity reduction not seen by our methods of hemispheric CBF measurement. Furthermore, measurement of neuronal conduction by recording evoked potentials might demonstrate functional improvement not detected by our morphologic measurements. Finally, our histologic studies were confined to the hippocampus where ischemic cell changes in this model have been shown to be reproducible and predictable. However, significant ischemic cell change also occurs in the cortex and striatum, and it is possible that we missed a positive morphological effect of viscosity reduction by not systematically examining those regions pre and post-exchange.

Conclusion

Isovolemic plasma exchange rapidly reduces fibrinogen and viscosity without affecting hematocrit or producing adverse neuropathological change. The magnitude of viscosity reduction correlated inversely with severity of cellular injury, and there was a modest improvement in CBF in treated animals. However, even if performed immediately upon reperfusion, fibrinogen reduction alone did not prevent ischemic injury in this model.

Further studies of viscosity reduction therapy in cerebral ischemia should include efforts aimed at reducing hematocrit and improving red blood cell deformability in addition to lowering fibrinogen concentration. Further studies using this particular animal model should examine the role of possible cytotoxic substances released at the time of ischemia, and should employ regional measurements of CBF, metabolism, and physiologic activity.

References

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