Forebrain Ischemia in the Rat

Relation Between Duration of Ischemia, Use of Adjunctive Ganglionic Blockade and Long-Term Recovery

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SUMMARY The relation between duration of ischemia, use of adjunctive ganglionic blockade and long-term recovery was studied in a rat model giving reversible subtotal forebrain ischemia. Ischemia was induced by bilateral carotid artery clamping and controlled hemorrhage to a mean arterial pressure of 50 mm Hg in animals artificially ventilated under 70% N₂O. After variable lengths of time, the clamps were removed and the drawn blood was reinfused. In some animals, the ganglion blocker Arfonad® was given (group A+) on induction of ischemia to facilitate hypotension. There was a strict dose-response relationship between duration of ischemia and mortality. Mortality was higher among animals not given Arfonad (group A−; 37% after 10 min of ischemia and 100% after 13 min) than in group A+ (about 20% after 12–13 min of ischemia, 50% after 15 min and 80% after 19 min). In group A+ more than half of the animals died later than 24 h after ischemia. All of them were hyperexcitable and 12% died during witnessed epileptic fits. Group A− animals regularly died within the first 24 h, with no indication of central nervous system involvement. Less blood had to be drawn to attain hypotension (mean arterial pressure 50 mm Hg) in group A+ (1.5 ± 0.3 ml/100 g b.w.) than in group A− (2.5 ± 0.2 ml/100 g b.w.). Group A+ also had less “washout” acidosis 5 min after reinfusion of the shed blood than group A− (15 min of ischemia: pH 7.24 ± 0.07 vs 6.96 ± 0.06). It is concluded that the model can be used reproducibly in studies on cerebral ischemia in the rat in which survival is required. If more than 10 min of ischemia is to be studied, Arfonad supplementation is necessary to avoid a general shock response to hemorrhage, which will contribute to mortality.

The obvious advantages of this model are that anesthetics and reversible cerebral ischemia in small animals is associated with considerable problems. This is reflected by the number of models and model modifications in use, which complicates direct comparisons between results from different laboratories. In the rat, essentially three different approaches are used to accomplish global cerebral ischemia for survival purposes. The four-vessel occlusion model is a two-step procedure in which the two vertebral arteries are first electrocauterized under ether anesthesia and reversible clamps are placed around the common carotid arteries. Twenty-four hours later, cerebral ischemia is induced in the awake rat by tightening the two carotid clamps. The obvious advantages of this model are that anesthesia is avoided, that hypotension is not required, and that the animals breathe spontaneously. However, owing to differences in collateral circulation the outcome for an individual rat is very unpredictable. A sizeable minority of the animals (about 30%) fail to lose consciousness after bilateral common carotid artery occlusion and some 8% (presumably those with most complete ischemia) die immediately as a result of respiratory failure. Reversible cerebral ischemia can also be established by infusing a pressure cuff around the animal’s neck under general anesthesia, combined with hemorrhagic hypotension. This causes very severe ischemia with a blood flow of less than 1% of the control in all parts of the brain. A method that has been used for more than a decade

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The four-vessel occlusion model is a two-step procedure in which the two vertebral arteries are first electrocauterized under ether anesthesia and reversible clamps are placed around the common carotid arteries. Twenty-four hours later, cerebral ischemia is induced in the awake rat by tightening the two carotid clamps. The obvious advantages of this model are that anesthesia is avoided, that hypotension is not required, and that the animals breathe spontaneously. However, owing to differences in collateral circulation the outcome for an individual rat is very unpredictable. A sizeable minority of the animals (about 30%) fail to lose consciousness after bilateral common carotid artery occlusion and some 8% (presumably those with most complete ischemia) die immediately as a result of respiratory failure.

Reversible cerebral ischemia can also be established by infusing a pressure cuff around the animal’s neck under general anesthesia, combined with hemorrhagic hypotension. This causes very severe ischemia with a blood flow of less than 1% of the control in all parts of the brain. A method that has been used for more than a decade is bilateral common carotid artery occlusion combined with hemorrhagic hypotension. To facilitate hypotension the ganglion blocker Arfonad® has recently been introduced as an adjunctive agent, which is administered on induction of ischemia. This model gives a very reproducible degree of cerebral ischemia. The blood flow to cortical structures, the hippocampus and the caudoputamen is below 5% of the normal, whereas more variable flows are recorded in subcortical structures such as the thalamus and hypothalamus. Until now, the model has mainly been used for studies on cerebral metabolism and blood flow during and after ischemia. In a recent communication, a way of adapting the model for long-term recovery studies is described. Before the model is used further in investigations of the efficacy of various treatment regimens, there is a need for systematic characterization of the relation between mortality and the duration of the ischemia and for an evaluation of the effect of Arfonad on the results. These matters have been dealt with in the present study.

Methods

Animal preparation

One hundred thirty-eight male rats of the Wistar strain (Møllegaard, Copenhagen) weighing between 300 and 400 g were used. The animals, five in each cage, were fasted overnight but had free access to tap water. The cages were equipped with a net floor to avoid coprophagia. After induction of anesthesia with 3% halothane in 70% N₂O and 30% O₂ the rats were intubated with a polyethylene tube (PE160) and connected to a Starling-type respirator (Braun, Melsungen AG). They were paralyzed with suxamethonium (3 mg/kg i.v.) and ventilated with 70% N₂/30% O₂ supplemented with 0.5% halothane during the surgical
procedure. Polyethylene catheters (PE50) were inserted into the tail artery and vein for continuous blood pressure recording, blood sampling and infusions. A catheter (PE60) was also inserted via the external jugular vein into the right atrium, and this catheter was only used to achieve the hemorrhagic hypotension. The animals were then given 300 IU of heparin per kg body weight (b.w.) i.v. The common carotid arteries were dissected free and loops of silk suture were placed around each vessel. EEG was recorded continuously via two gold needles inserted through small incisions below the galea. The body temperature was continuously monitored with a rectal thermometer and kept close to 37°C by external heating.

After completion of the surgical procedure, the halothane supply was discontinued and the animals were allowed a steady state period of 30 min during which the acid-base status was checked (pH/blood gas analyzer, IL 1302) and the ventilation was set to obtain values within the following ranges: pH 7.35–7.40; pCO2 4.67–5.50 kPa and pO2 11.0–18.0 kPa. All animals not fulfilling these criteria at the end of the steady state period or in which the initial blood glucose level lay outside the range 3–8 mmol/l were excluded from the experiments.

Experimental Protocol

The animals were divided into two main groups. Those of the first group (group A+) were given trimetaphan camphor sulfonate (Arfonad®; Hoffman-La Roche, Basel, Switzerland), a ganglion blocking agent, i.v. until until a mean arterial blood pressure (MAP) of 80 mm Hg was reached. The carotid arteries were then clamped and more Arfonad was given to a total dose of 15 mg/kg b.w. This was followed by controlled hemorrhage through the central venous catheter to MAP of 50 mm Hg. The hemorrhagic period was not allowed to exceed 2 min and the beginning of cerebral ischemia was defined as the time when the EEG became isoelectric. The shed blood was kept at a predetermined distance from the head to maintain the temperature of the brain at 37°C during the ischemia. At the end of the ischemic period, shed blood was infused at MAP of 80 mm Hg, the clamps were released. When the animals started to breathe spontaneously, usually 50–70 min after termination of ischemia, they were ventilated with 100% O2 for 2 min and then disconnected from the ventilator and given 5 ml of 0.9% NaCl subcutaneously. They were extubated either when they no longer tolerated the tracheal tube or when they showed sufficient spontaneous respiration.

In the second group (group A−) forebrain ischemia was induced without the use of Arfonad. At the end of the steady state period these animals were bled through the central venous catheter until MAP of 80 mm Hg was reached, the two common carotid arteries were clamped and the bleeding was rapidly continued to MAP of 50 mm Hg, whereafter the two groups were treated identically.

Animals were kept hypotensive with the carotid arteries clamped and with isoelectric EEG for 10, 12, 13, 15, 17, 19 or 20 min (see Results).

In all animals blood gas, blood glucose and hematocrit were checked before induction of ischemia, at the end of the ischemic period, and 5, 30 and, if not yet decatheterized, 60 min after the start of recirculation. MAP and EEG were recorded continuously.

Nothing was done actively to correct the physiological derangements after ischemia. Food and water were placed on the cage floor and also at the usual positions to facilitate nutrition. Every second day the animals were weighed.

The experimental protocol was accepted by the Regional Ethical Committee of the University of Uppsala.

Statistics

Differences between groups in mortality at various times were evaluated by conventional life table analysis, corrected chi-squared or Fischer's exact test. For other statistical comparisons between groups Student’s t-test was used. Data in the text and figures are given as mean ± S.D.

Results

Figure 1 (left) shows the blood pressure recording in a typical experiment without Arfonad, i.e. in group A−. At B1, the hemorrhage was started and at C the two common carotid arteries were clamped. As an effect of the clamping, there was an intense pressor response, the blood pressure increased and the hemorrhage was rapidly continued at B2. At this moment groups A+ and A− reacted differently. The increase in blood pressure was less pronounced in group A+ (fig. 1 right) and a smaller amount of blood had to be withdrawn to reduce MAP to 50 mm Hg than in group A− (1.5 ± 0.3 ml/100 g b.w. v 2.5 ± 0.2 ml/100 g b.w.). Both factors contributed to the shorter interval between clamping and isoelectric EEG in group A+ (41 ± 19 s) than in group A− (71 ± 25 s; p < 0.05).
At D the clamps were removed after blood had been reinfused to MAP of about 80 mm Hg, whereafter the reinfusion was completed at R. In brief, it was technically more difficult to obtain a "square wave" ischemia when the hemorrhagic hypotension was not supported by active vasodilation.

MAP recovered in a similar way in the two experimental groups and was about 125 mm Hg 5 min after reinfusion, with no difference between animals that were dying and those that were not (data not presented).

EEG recovery was also similar in animals given Arfonad and those subjected to hemorrhage alone, both in survivors and in non-survivors (data not presented).

Most of the animals could be disconnected from the ventilator 60 min after termination of ischemia and were extubated after another hour. During this period they were comatose but subsequently they usually started to show spontaneous movements. During the first 24 to 48 h most of the animals displayed signs of neurologic damage. They were inactive and more or less lethargic. When stimulated, however, they were hyperexcitable and sometimes responded with one or more jerks. A few animals were hyperactive, moving continuously around in the cage. One of the more serious signs which often predicted later death, was a unidirectional circling form of behavior and meaningless movements of the front legs. Most animals started to drink after 2 days and to eat after 2–4 days. Their body weights declined during the first 3–6 days and the preoperative weight was not regained until 10–14 days after the insult.

Animals that were given Arfonad on induction of the ischemic insult seemed to have a better outcome than those not given this ganglion blockier (fig. 2). After 10 min of ischemia all the animals of group A + survived, in contrast to a 37% mortality 96 hours after the insult in group A − (p < 0.10). After 12 min of ischemia the mortality in group A − was 70%, compared with 25% in group A + (p < 0.10) and after 13 min these figures were 100 and 18% respectively (p < 0.001). Among animals given Arfonad the mortality was 54% and 77% mortality after 17 and 19 minutes of ischemia respectively. Even after 20 min of ischemia it did not reach 100% in animals which received Arfonad.

Treatment with Arfonad had an impact not only on the overall mortality but also on the time of death for a given mortality. Comparison of survival curves were performed using the Mantel-Cox and Breslow tests. There were highly statistically significant differences in shape between survival curves in favour of Arfonad®-treated animals after 12, 13 and 15 minutes of ischemia (p < 0.05, p < 0.001, p < 0.001). At 10 minutes p was approximately 0.05. Of the 23 animals that died within 2 h after the insult, only six were given Arfonad and 17 were not, whereas 40 of the 49 animals that died after this time point belonged to group A + (p < 0.001, chi-square). Fourteen of the 32 animals dying within 6 h, but 32 of the 40 animals that died after 6 h, were Arfonad-treated (p < 0.01, chi-square). This effect of administration of Arfonad is also evident from a comparison of life tables for Arfonad-treated animals after 19 min of ischemia and non-Arfonad-treated animals after 12 min of ischemia. Both groups showed an overall mortality of about 75%, but in the group not given Arfonad the risk of dying within 2 hours and 6 hours was higher (50% v 8%, p < .02, and 30% v 8%, p < 0.001; life table analysis).

None of the 23 animals which died within 2 h after termination of the ischemia exhibited any seizure activity, but some of them had a gasping respiration and peripheral cyanosis prior to death. Twenty animals died between 2 and 24 h after termination of the ischemia, often non-witnessed during the night after the experiment. In some of these, findings in and around the cage suggested terminal seizure activity. The 29 animals which died later than 24 h post-ischemia all exhibited marked hyperexcitability and in 12 of them terminal seizures were observed. Terminal seizures

![Figure 2](http://stroke.ahajournals.org/)

**FIGURE 2.** Cumulative mortality with time after various durations of cerebral ischemia. — = group A +; ----- = group A −. Each point represents the death of one animal. Figures in brackets indicate number of animals in each group.
were seen in increasing frequency with longer durations of ischemia (1 out of 13, 4 out of 14 and 6 out of 10 after 15, 17 and 19 min of ischemia respectively). Some surviving animals suffered one or more epileptic convulsions and then behaved normally, but as a rule the occurrence of epileptic fits indicated that the animal would die within 6–8 h.

Autopsy was performed on all animals whose death was witnessed, with the main purpose of investigating whether gross morphologic alterations in the respiratory or circulatory system could have contributed to death. Dead animals from group A — were found to have pale, swollen kidneys with a hemorrhagic zone at the cortico-medullary junction, a patchy liver, and hemorrhagic necrosis of the intestinal mucosa. These changes were more pronounced with longer periods of ischemia, but were never observed in animals treated with Arfonad.

After the ischemia non-survivors generally showed a more marked decrease in pH and in base excess (BE) than survivors (data not shown). These observations were made after shorter periods of ischemia in animals not given Arfonad and after longer periods of ischemia in Arfonad-treated animals. The patterns of glucose concentration, hematocrit and pCO₂ were similar in non-survivors and survivors, apart from values found at a few isolated observation points.

The changes in systemic variables with time were different in Arfonad-treated and non-Arfonad-treated animals. These differences, which were observed with all durations of ischemia, are depicted for the 15-min ischemia groups in figure 3. In group A — there was pronounced metabolic acidosis at the end of the ischemic period, seen as a very low pH (7.24 ± 0.14) and a negative base excess (−15.9 ± 3.1). After 5 min of recirculation the systemic acidosis was even more profound (pH 6.96 ± 0.06 and BE −21.1 ± 1.9) and after 60 min of recirculation some restoration had occurred. The alterations in group A + were considerably milder and both pH and BE were significantly less deranged than in group A — at the end of the ischemia and throughout the observation period. Owing to the constant respiration, during ischemia there was a decrease in pCO₂, which was more pronounced in group A — than in group A +. The blood glucose level increased in group A + during ischemia (fig. 3), but did not change in group A —. During recirculation it remained high in group A +, but declined in group A —.

As expected, the hematocrit decreased during ischemia, to a greater extent in group A +, and increased again on recirculation. In group A + it then remained lower than the steady state value, while in group A — there was a tendency to hemoconcentration.

**Discussion**

Forebrain ischemia in the rat induced by bilateral occlusion of the common carotid artery and simultaneous hemorrhagic hypotension has previously been used extensively in metabolic studies of postischemic brain damage. Not until recently was this model adapted to allow survival of the animal. In the present study we have established the existence of a close relationship between duration of ischemia and postischemic mortality. We have also demonstrated the importance of using pharmacologic vasodilation as an aid to induction of hypotension in order to avoid serious systemic circulatory derangements, which in themselves contribute to mortality.

Our results fit well with the data obtained by Smith and coworkers, but these authors did not systematically investigate the relation between duration of ischemia and mortality. They reported a slightly higher mortality than our figure after 15 minutes of ischemia in Arfonad-treated rats (75% versus 50%). In their hands administration of Arfonad did not influence the intensity of the cerebral ischemia as such, the electrophysiological recovery after ischemia, MAP during recirculation, or regional cerebral blood flow 90 minutes after termination of the ischemia.

They found that Arfonad blunted the increases in plasma adrenaline and noradrenaline concentrations which occurred during and after ischemia. In our study the maximal amount of blood that had to be drawn to reduce MAP to 50 mm Hg was smaller in Arfonad-treated rats than in those not given Arfonad and the “washout” metabolic acidosis seen immediately after resumption of normal circulation was also less pronounced in animals that received Arfonad. This, considered together with the findings of Smith and coworkers, shows that administration of Arfonad counteracts both the sympathoadrenal response and the systemic effects of this response.

The occurrence of more early deaths — within a few hours after the insult — in animals not given Arfonad, combined with the morphologic findings of intestinal mucosal necrosis and pale, swollen kidneys with a hemorrhagic zone in the deep cortex, seems to indicate that systemic factors of a “shock nature” rather than the
primary brain damage were responsible for the difference in mortality between Arfonad-treated and non-Arfonad-treated animals.

If hemorrhagic shock contributed to the mortality in A—animals, as the results seem to suggest, it is thus reasonable to suppose that the bilateral carotid artery occlusion as such potentiated the systemic response to hypotension. In experimental hemorrhagic shock, hypotension lasting more than 45 minutes is required to induce shock of such a degree as to lead to certain mortality.12 13 A reasonable explanation would seem to be that the baroreceptor response induced by the bilateral carotid artery clamping, and the pressure response to the cerebral ischemia together exert a considerable degree of extra sympathetic stress on the animals, thus potentiating the circulatory derangements caused by the hemorrhagic hypotension itself. In other words, after carotid artery clamping and hemorrhagic hypotension, the animals in fact suffer more serious circulatory derangement than might be expected from the degree of hypotension as such. Another possibility is that centrally controlled protective mechanisms involved in the resistance to hemorrhagic shock14 are interfered with as a result of the cerebral ischemia. One example of such a possible mechanism is the hypotension-induced and hypothalamus-controlled release of vasopressin,15 to which opioid peptides seem to contribute.16

Fifteen minutes of cerebral ischemia in Arfonad-treated animals was followed by 50% mortality, most of the animals dying more than 24 hours after the insult with a clinical picture indicating that their death was of neurogenic origin. In the four-vessel occlusion model of Pulsinelli,17 20 minutes of ischemia in nonanesthetized animals, without hypertension, was followed by a mortality of only 8%. The reason for the discrepancy between this result and ours is not clear. The anesthesia and the hypotension required in our model, in contrast to that of Pulsinelli, and a difference in the degree of ischemia are three possible explanations.

Although the nitrous oxide anesthesia as such causes only minor alterations in cerebral blood flow and glucose uptake,18 19 direct comparison between results obtained in awake and anesthetized animals is not justifiable.

Regulation of blood pressure involves a complex neuroanatomic and functional organization. This integrated system promptly responds to peripheral stimuli, above all from the carotid sinus and the aortic depressor nerves,20 and even limited hypotension in the rat is quickly followed by enhancement of glucose metabolism in certain brain areas.21 A direct comparison between ischemic brain damage induced in hypotensive and normotensive animals therefore seems inappropriate.

The two-vessel occlusion model used in our study results in virtual cessation of flow in anterior cortical structures, while the flow in the basal nuclei is up to 30–40% of the normal, and that in the brain stem is even higher.8 22 The cortical lactate concentration reaches about 15 μmol/g tissue. The flow reduction in the Pulsinelli model seems to be more complete and is followed by a lower accumulation of lactate (about 10 μmol/g tissue) in the ischemic cerebral cortex.23 24 Whether this difference in lactate accumulation can explain the discrepancies in mortality is not known, but this explanation seems less probable.

The existence of a strict dose-response relationship between duration of ischemia and mortality shows that this model can be a useful tool in the evaluation of long-term consequences of various types of treatment after ischemia. If the model is used in that context, Arfonad supplementation should be the rule.

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