Multiple, repetitive hypoxic or ischemic brain insults may occur in a variety of clinical circumstances such as transient ischemic attacks, surgical procedures, arrhythmias, or during the intrapartum period. In experimental models, brief repetitive periods of cerebral ischemia have been shown to be more damaging than a single period of cerebral ischemia of similar total duration. The mechanism(s) responsible for this exacerbation of cerebral injury is unknown; however, several possibilities exist, including progressive accumulation of cytotoxic substances such as free radicals, prostaglandins, and excitatory neurotransmitters or exaggerated alterations of cerebral blood flow.

Although it is known that prostaglandins accumulate during ischemia and reperfusion, the significance of these changes is unknown. In an effort to further characterize the model of repetitive ischemia, we documented the time course of changes in brain water and sodium caused by single and repetitive periods of cerebral ischemia and also compared the degree of edema caused by each type of insult. In addition, some animals were pretreated with the cyclooxygenase inhibitor indomethacin in an attempt to determine if excessive accumulations of prostaglandins might mediate the more severe edema seen after repetitive ischemia.

Materials and Methods

One hundred fifteen adult male gerbils (Meriones unguiculatus), mean weight (±SEM) 74±1 g (Tumblebrook Farms, West Brookfield, Mass.), were used in the study. Surgical procedures and general care were conducted in accordance with the University of Michigan principles for the care and use of laboratory animals.

The gerbils were divided into one control group and four experimental groups. Control animals (n=6) were killed and processed as described below,
Immediately after induction of anesthesia, without femoral or neck surgery. The experimental groups I–IV were treated as follows: group I received a single 15-minute period of bilateral carotid occlusion after pretreatment with 0.9% saline, group II underwent three 5-minute periods of bilateral carotid occlusion at hourly intervals after pretreatment with 0.9% saline, group III received a single 15-minute period of bilateral carotid occlusion after pretreatment with 0.2 mg/kg i.v. indomethacin (Merck Sharp & Dohme) dissolved in 0.9% saline 15–20 minutes before ischemia, and group IV underwent three 5-minute periods of bilateral carotid occlusion at hourly intervals after pretreatment with 0.2 mg/kg i.v. indomethacin in 0.9% saline 15–20 minutes before ischemia.

After weighing, anesthesia was induced by 2% halothane with 70% N₂O/30% O₂. Previous experiments had documented that this level of anesthesia did not result in hypotension. Body temperature was maintained between 36–38°C using a servo-controlled warming pad and rectal temperature probe. In experimental gerbils, the left femoral vein was cannulated with PE-10 tubing followed by injection of saline or indomethacin (0.2 mg/kg) each at a volume of 2 ml/kg body wt. The catheter was then removed, the femoral vein cauterized, and the incision sutured. After completion of the femoral surgery, the anesthesia was decreased to 0.5% halothane and 70% N₂O/30% O₂.

The carotid arteries were approached by a midline anterior cervical incision. Each carotid artery was carefully isolated from surrounding nerves and veins. After isolation, a loose 5-0 ligature was placed around each vessel for ease in handling. Weck microaneurysm clips were placed on the left carotid and then the right. The interval between occlusion of the left and right carotid arteries ranged from 5 to 15 seconds. Cessation of blood flow was visually confirmed in all cases. After the specified time, the clips were removed and reestablishment of flow was confirmed visually. The skin incision was sutured and the anesthesia was stopped. The gerbil remained supine on the warming blanket until awake enough to right itself, at which time it was placed in a cage.

Gerbils requiring multiple carotid occlusions were reanesthetized with halothane/N₂O/O₂ just before carotid reocclusion. The carotids were occluded in a similar fashion 1 hour after the previous occlusion. Animals in groups requiring only a single 15-minute carotid occlusion underwent two further periods of anesthesia at hourly intervals but without carotid occlusion to control for possible effects of repeated anesthesia.

Nine gerbils underwent sham surgery to mimic repetitive occlusion. After femoral vein cannulation, neck surgery was performed. The carotid arteries were isolated and manipulated, but no occlusion was performed. This was repeated along with anesthesia a total of three times. Five animals were pretreated with saline and four with indomethacin. All were killed 48 hours after surgery.

Experimental animals (groups I–IV) were killed by decapitation 24, 48, or 72 hours after the final carotid occlusion. Each gerbil was placed in an ether jar for 30–45 seconds before being killed. Control gerbils were decapitated immediately after induction of anesthesia. Each brain was removed from the cranium, and the brain stem was removed and discarded. The cerebellum was removed and placed in a preweighed crucible. The cerebral hemispheres were divided in the sagittal plane, and the cortex was separated from the deep white matter with curved forceps. Each cortex was placed in a preweighed crucible. After determination of the wet weights, each sample was baked at 100°C for 24 hours and the dry weight measured. Percent tissue water was then calculated for the cortex and nonischemic cerebellum. The desiccated tissue was then ashed at 400°C for 16 hours, and the sodium content of each tissue region was determined by flame photometry. After determination of percent tissue water and sodium content, the results obtained from each hemicortex of an individual gerbil were combined before statistical analysis.

After the brain water and sodium contents of each brain region at 24, 48, and 72 hours were determined, single-factor analysis of variance was used to compare results of each group to control values. A value of p=0.05 was used to assign significance. Two-tailed Student's t tests with Bonferroni's correction were used to compare given parameters between different experimental groups at 24-, 48-, and 72-hour time points (group I versus II, group I versus III, and group II versus IV). Fisher's exact test was used to compare mortality related to type of ischemic insult and type of pretreatment.

Results

Following a single 15-minute period of ischemia after saline pretreatment (group I), the percent water (±SEM) of the cortex increased from 79.10±0.22% (control) to a maximum of 80.14±0.46% at 48 hours, a change that did not reach statistical significance. Cortical sodium content after this insult increased significantly from 206±2 in control cortices to 258±11 meq/kg dry wt at 48 hours (p<0.017).

In contrast to single ischemia, a marked increase in both cortical water and sodium content occurred in gerbils after multiple periods of ischemia (group II) as compared with control values, with cortical brain water reaching a maximum of 82.15±0.31% (p<0.0001) and cortical sodium content reaching 420±14 meq/kg dry wt (p<0.0001). The peak of each measurement occurred 48 hours after the insult in both groups.

Gerbils undergoing a single period of ischemia and that were pretreated with indomethacin (group III) showed changes in cortical water and sodium content...
that were similar to those seen in gerbils undergoing a similar insult but that were pretreated only with saline (group I). Group III gerbils did not show an increase in brain water compared with control values (peak value, 79.70±0.18%), but had significantly increased cortical sodium content (peak value, 242±7 meq/kg dry wt, \( p=0.026 \)).

Gerbils undergoing repetitive ischemia after pretreatment with indomethacin (group IV) showed significant increases in both cortical water (peak value, 80.90±0.36%, \( p=0.005 \)) and sodium content (peak value, 355±36 meq/kg dry wt, \( p=0.03 \)) compared with control values. The values for these measurements changed minimally from 24 to 72 hours after insult.

Sham-operated gerbils pretreated with saline or indomethacin and killed 48 hours after surgery showed no change in cortical water content (79.05±0.07% and 79.07±0.07%, respectively, versus 79.10±0.22%, control). No change was noted in cortical sodium content of saline pretreated sham-operated gerbils (216±4 meq/kg dry wt), and a modest, but significant, increase was seen in indomethacin-treated, sham-operated gerbils (225±7 versus 206±2 meq/kg dry wt, control, \( p=0.01 \)).

Comparisons at individual time points between groups undergoing single and repetitive ischemia after saline pretreatment (Figure 1A) showed that brain water was significantly higher after multiple ischemic periods (group II) than after a single ischemic period (group I) both 24 and 48 hours after the insult (\( p=0.008 \) and 0.005, respectively). There was no significant difference in brain water between groups I and II at 72 hours. Similarly, cortical sodium content (Figure 1B) was significantly higher in gerbils that underwent three 5-minute periods of ischemia (group II) rather than a single 15-minute period of ischemia (group I) both 24 and 48 hours after ischemia (\( p=0.002 \) and 0.0001, respectively).

The cerebellum, which is not made ischemic in the gerbil model of bilateral carotid occlusion, had water contents ranging from 76.28% to 77.20% and sodium contents ranging from 177 to 194 meq/kg dry wt in groups I and II (Table 1). None of these values differed significantly from the control values of 76.70±0.26% and 181±3 meq/kg dry wt, nor were there differences between groups at any time point after ischemia.

As previously stated, single ischemia with saline pretreatment resulted in no significant change from control in cortical water content, but a modest, though significant, increase in cortical sodium content. Comparison of groups receiving saline or indomethacin before a single 15-minute period of ischemia revealed no significant differences between groups I and III in either cortical water or sodium content at any time point after ischemia (Figure 1).

There were no differences in cerebellar water content between group I and group III gerbils at any time point after ischemia, nor did any of these values differ from control. As noted, the sodium content of the cerebellums of group I gerbils did not differ from control. Sodium content of the cerebellums of group III gerbils differed from control values (Table 1) only at the 24-hour time point (\( p=0.01 \)). This value was also significantly higher than the corresponding value in group I (220±9 versus 194±2 meq/kg dry wt, \( p=0.015 \)).

Comparison of gerbils undergoing repetitive ischemia (Figure 1) that received indomethacin (group IV) to those pretreated with saline (group II) revealed significantly less brain water (80.02±0.45 versus 82.15±0.31%, \( p=0.003 \)) and lower sodium content (300±39 versus 420±14 meq/kg dry wt, \( p=0.016 \)) 48 hours after the insult in the gerbils pretreated with indomethacin. Differences at 24 and 72 hours were not significant.

The values for cerebellar water and sodium content (Table 1) in gerbils from groups II and IV did not differ from control values, nor were there significant differences between the two groups at any time point.

Cerebellar water content of animals undergoing sham surgery after saline or indomethacin treatment showed no change in water content (76.99±0.20 and 76.80±0.09, respectively, versus 76.70±0.26, control).
Cerebellar sodium content was slightly elevated in both these groups of gerbils (202 ± 7 and 203 ± 8, respectively, versus 181 ± 3 meq/kg dry wt, control, p ≤ 0.02).

Mortality after a single 15-minute ischemic insult (those dying before the 48- or 72-hour time point) was 6.9% (two of 29 animals). There was no difference based on pretreatment with saline or indomethacin (zero of 12 versus two of 17, p = 0.34, Fisher’s exact test). Mortality after repetitive ischemia was 25% (nine of 36), also with no significant difference noted after saline or indomethacin pretreatment (three of 15 versus six of 21, p = 0.43, Fisher’s exact test). The difference in mortality between gerbils undergoing single or repetitive ischemia (combining saline- and indomethacin-treated gerbils) was significant (two of 29 versus nine of 36, p = 0.05, Fisher’s exact test). Comparison of mortality based only on treatment with saline or indomethacin showed no significant difference (three of 27 versus eight of 38, respectively, p = 0.24, Fisher’s exact test).

**Discussion**

Our results indicate that brain edema resulting from three 5-minute periods of cerebral ischemia spaced at hourly intervals peaks approximately 48 hours after the insult. The time course of edema seen after a single 15-minute period of cerebral ischemia is similar, but the change in the percentage of cortical water did not reach statistical significance and thus the presence of edema is not clearly documented. However, the increased sodium content in the cortex after a single ischemic insult was significant and paralleled the increase in water content, suggesting that edema was present after 15 minutes of ischemia. Both water content and sodium accumulation in the cortex were significantly greater after repetitive ischemia.

Although cortical sodium and water content peaked 48 hours after the ischemic insult, the level and timing of the peak may be misleading. After repetitive ischemia, 25% of the gerbils died before 48 or 72 hours and thus were not included in the determination of edema or sodium content. Animals that died after an ischemic insult might reasonably be expected to be more severely affected than the survivors. Thus, the peak in edema and sodium content seen at 48 hours might have been higher and the subsequent decline less rapid after repetitive ischemia if the values for brain water and sodium content of gerbils that died could have been included.

Similarly, higher mortality in the gerbils undergoing repetitive ischemia that received indomethacin could artificially lower the mean values for brain water and sodium content compared with repetitive ischemia animals pretreated with saline. As noted above, mortality was not significantly different in the repetitive ischemia gerbils based on saline or indomethacin pretreatment. Nonetheless, the fact that the mortality was slightly higher after indomethacin pretreatment (28.6% versus 20%) may have lowered the mean values for water and sodium content in these gerbils to some degree.

As expected, the nonischemic cerebellum did not show significant water accumulation at any time point in any group. The magnitude of the increase in the cerebellar sodium content at 24 hours in gerbils subjected to 15 minutes of ischemia after indomethacin pretreatment (group III) was small in comparison to changes in the cortex. Similarly, gerbils that underwent sham surgery showed no changes in cortical or cerebellar water content but tended to show slight increases in sodium content. These were small in comparison to cortical changes after repetitive ischemia. We believe these to be of unlikely biological significance despite their statistical significance; however, a minor effect of surgery or anesthesia is also possible.

These data confirm and expand on data reported previously. Tomida et al documented greater damage histologically, lower tissue specific gravity, and higher mortality 24 hours after insult in animals that underwent repetitive ischemia as opposed to a single...
period of ischemia. Our data confirm that there is increased brain edema in animals that persists beyond 24 hours after repetitive ischemia. The most severe edema occurs 48 hours after insult.

Gerbils subjected to 15 minutes of ischemia and pretreated with saline or indomethacin had no notable differences in cortical edema or sodium content. Both groups had insignificant elevations of cortical water, but significant increases in cortical sodium content. In view of the marginal degree of edema, it is perhaps not surprising that no discernable effect was found with indomethacin pretreatment. In contrast, in gerbils subjected to repetitive ischemia, indomethacin pretreatment resulted in a marked decrease in the amount of edema and sodium accumulation at the 48-hour time point in comparison to saline-treated gerbils.

The mechanism by which indomethacin lessens cerebral edema after repetitive ischemia is unclear. Indomethacin has been used to lessen postischemic cerebral edema in other animal models where it has been effective during partial ischemia and ischemia with reperfusion, suggesting that arachidonic acid and prostaglandins are at least partially responsible for edema seen after ischemia. The level of arachidonic acid, the major substrate for prostaglandin synthesis, increases during brain ischemia and several prostaglandins have been shown to increase during postischemic reperfusion. Prostaglandins, particularly of the E and I series, are well known to affect capillary permeability and cause vascular dilatation. Thus, increases in the levels of these prostaglandins may lead to capillary leakage and edema. These parameters have not been studied in the context of repetitive ischemia; however, the effect of indomethacin on edema after repetitive ischemia suggests that prostaglandins are involved.

Mechanisms involving prostaglandins by which repetitive ischemia could cause increased brain injury are speculative. As suggested by Tomida et al, repetitive periods of ischemia that occur during postischemic hypoperfusion may be critical prerequisites for aggravated cerebral injury. The levels of some prostaglandins appear to peak in brain tissue after only a few minutes of reperfusion and to decrease within 15 minutes after ischemia. Levels in the cerebrospinal fluid peak at varying times with dilator prostaglandins peaking early in reperfusion and constrictors later, resulting in a predominance of constrictor prostaglandins. This may be significant with repetitive ischemia. Ischemia occurring at the time of constrictor prostaglandin predominance may further aggravate this imbalance between constrictor and dilator prostaglandins. Ischemic periods at hourly intervals occur during the hypoperfusion or presumed constrictor predominance period.

Conversely, a balance between dilator and constrictor prostaglandins may also explain why repetitive ischemia with short interischemic periods (i.e., 3-10 minutes) does not appear to cause damage as severe as that seen with interischemic intervals of 60 minutes. Ischemia commencing during the vasodilator prostaglandin predominance phase (hypemic phase) might have a different effect on the relationship of constrictor and dilator prostaglandins. To our knowledge, these possibilities have not been examined in the model of repetitive cerebral ischemia. Based on the scheme outlined above, one might expect that indomethacin treatment before ischemia may affect postischemic cerebral hemodynamics. Indomethacin has been used in animal models to ameliorate postischemic hypoperfusion. Repetitive ischemia results in hypoperfusion after each period of cerebral ischemia that does not appear to be more severe than that seen after a single equivalent period of ischemia; however, the reactive hyperemia seen immediately after each successive period of ischemia appears less pronounced. The lessened hyperemia may impede the reestablishment of flow in previously ischemic areas that are blocked because of platelet clumps or swelling of capillary endothelium. The successively lower degree of reperfusion might result in progressively enlarging areas of no-reflow and presumed continued microvascular ischemia. Vass et al correlated abnormalities of the microcirculation after repetitive ischemia with brain tissue edema. Thus, if indomethacin improves flow during the hyperemic phase or lessens the degree of later hypoperfusion, this later persistent and widening area of ischemia after repetitive ischemia may be prevented.

Other mechanisms not directly dependent on prostaglandin levels may be involved. Free radicals, which have been implicated in the development of ischemic brain edema, are generated via the cyclooxygenase pathway. If arachidonic acid is released with each successive ischemic insult, further generation of free radicals will occur as arachidonic acid is removed via the cyclooxygenase pathway, possibly inducing further damage. Prevention of repetitive release of these reactive molecules by cyclooxygenase inhibition may account for the effects of indomethacin.

Indomethacin might also exert effects by affecting brain temperature in the peri-ischemic period. Small differences in brain temperature are known to affect the extent of cerebral injury after ischemia and transient hyperthermia has been noted to occur in animals after cerebral ischemia. Repeated bouts of cerebral hyperthermia after ischemia, which were undetected by core temperature monitoring, could account for more severe damage occurring after repetitive ischemia. Indomethacin has known antipyretic effects and by suppressing temperature elevations might decrease cerebral injury. This possibility, along with those previously mentioned, will require further investigation, both to delineate the mechanism(s) of increased injury with repetitive ischemia and of its amelioration by indomethacin.

In summary, our results indicate that edema after repetitive cerebral ischemia may be secondary to changes in prostaglandin metabolism and suggest that differences in prostaglandin metabolism are...
responsible for the differences in edema seen after single or repetitive cerebral ischemia. Other mechanisms are also possible, thus comparison of prostaglandin levels and the effects of indomethacin on postischemic perfusion and brain temperature in this repetitive ischemia model may provide important information concerning the mechanisms of ischemic brain injury.

References

KEY WORDS • indomethacin • cerebral ischemia • brain edema • gerbils
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