Cerebral Hypoperfusion and Delayed Hippocampal Response After Induction of Adult Kaolin Hydrocephalus

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Background and Purpose—In chronic hydrocephalus, a role for tissue hypoxia resulting from cerebrovascular compression is suggested. The purpose of this study was to evaluate whether changes in cerebral blood flow (CBF) in the time course of adult kaolin-induced hydrocephalus correlated with immunohistochemical neuronal responses.

Methods—In 46 adult Sprague-Dawley rats, kaolin hydrocephalus was induced and immunostaining of neurofilament protein (NF68), synaptophysin (SYN38), and neuronal nitric oxide synthase (NOS) was performed at 2 (short term), 4 (intermediate term), and 6 and 8 (long term) weeks. Local CBF was measured quantitatively by [14C]iodoantipyrine ([14C]IAP) autoradiography in the short-term stage and in both long-term stages.

Results—At 2 weeks, neuronal NOS immunoreactivity was globally increased in cortical areas and within the hippocampus. Four weeks after hydrocephalus induction, a reactive increase of SYN38 and NF68 immunoreactivity in the periventricular cortex was seen. At 6 and 8 weeks, when the ventricular size was decreasing, immunohistochemical changes in the hippocampus became most evident. A maintained toxic NOS reactivity in the CA1 subfield was accompanied by a loss of NF68 staining. In the CA3 subfield, however, focal increases in NF68 and SYN38 immunoreactivity were found. Cortical and hippocampal blood flow showed prolonged decreases of 25% to 55% compared with control animals. At 8 weeks, control levels were reached.

Conclusions—The observed temporary CBF decrease appears to correlate with an early global neuronal ischemic response. In addition, it may also account for the delayed selective response of ischemia-vulnerable structures, eg, hippocampus, in chronic adult kaolin-induced hydrocephalus. (Stroke. 2003;34:193-199.)

Key Words: autoradiography ■ cerebral ischemia ■ hippocampus ■ hydrocephalus ■ rats

Recent investigations in experimental kaolin-induced hydrocephalus have shown varying involvement of cholinergic, dopaminergic, and noradrenergic neurotransmitter systems, indicating a rather complex neuronal disturbance.1–4 Cerebral ischemia was suggested as a potential cause for the observed neuronal dysfunction by investigators who measured cerebral metabolism using magnetic resonance spectroscopy.5

Previous cerebral blood flow (CBF) investigations in which the H2-clearance method and the [14C]iodoantipyrine ([14C]IAP) autoradiography technique were used showed that cortical gray matter blood flow sustained rather mild decreases in adult and infantile experimental hydrocephalus.6 Only blood flow levels in the white matter were found to be below the ischemic threshold.7 Therefore, the suggested relationship between diminished CBF and the observed neuronal changes in hydrocephalus is not clear.5

In the present study we sought to measure CBF and investigate the neuronal responses in the time course of adult experimental kaolin-induced hydrocephalus. Local CBF was quantified with the use of [14C]IAP autoradiography and was correlated with the immunohistochemical findings at the neuronal cellular level.

Materials and Methods

Induction of Kaolin Hydrocephalus

The study observations are based on forty-six 10-week-old adult male Sprague-Dawley rats (weight, 300 to 420 g) that were randomized for hydrocephalus induction. An additional 23 unoperated animals served as controls that were held under equal feeding and room conditions for 2 weeks. The study was approved by the Bezirksregierung, Hannover, Germany. Anesthesia was induced by intraperitoneal injections of ketamine (100 mg/kg) and xylazine (8 mg/kg). Rats were allowed to breathe spontaneously while anesthesia was maintained by supplemental doses of ketamine (25 mg/kg).

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After microsurgical exposure of the atlanto-occipital membrane, a 20-gauge needle was inserted into the subarachnoid space. Then 0.1 to 0.2 mL of CSF was withdrawn, and an equal quantity of kaolin suspension (0.0375 g aluminum silicate diluted with 0.9% saline) was slowly injected. Rats were weighed twice per week. All animals exhibited transient signs of lethargy and episodes of limb ataxia. No focal neurological deficits were seen.

**Histological and Immunohistochemical Studies**

Histological studies were performed on 28 hydrocephalic rats at 2 (n = 5), 4 (n = 8), 6 (n = 9), and 8 (n = 6) weeks. The hydrocephalic stages were assigned as follows: short term (2 weeks), intermediate term (4 weeks), and long term (6 and 8 weeks). Seven animals served as controls.

For histological processing, animals were killed with an overdose of barbiturate, and the brains were quickly removed, covered with a cryomatrix, and frozen to −70°C in isopentane chilled with liquid nitrogen. Cryosections of 5-μm thickness were labeled with primary antibodies to neurofilament protein 68 kDa (NF68; monoclonal, 1:1000, Sigma), synaptophysin 38 kDa (SYN38; monoclonal, 1:20, DAKO), and neuronal nitric oxide synthase (NOS; polyclonal, 1:400, Sigma) with the use of the horseradish peroxidase–coupled secondary antibodies. Specificity controls included the omission of the primary antibody.

Except for immunostaining of NOS, which is normally expressed only in a small population of neurons,8 image analysis with the use of a National Institutes of Health program (Scion Image) was performed to quantify immunostaining. The sections were analyzed with the aid of a photomicroscope (Zeiss) with a ×40 objective and digital capture. The originally captured true color image was converted into an RGB (red, green, blue) image. The blue channel was converted to gray scale (0 to 255), and density slicing was performed to distinguish between stained (objects) and unstained (background) tissue. After conversion to binary, the quotient of the number of black pixels (objects) divided by the number of white pixels (background) within the measured area (microscopic view at ×40 magnification) was referred to as “immunoreactivity.”

The regional measures of 2 serial sections were taken from the hippocampal CA1 and CA3 subfields, including the stratum oriens and radiatum and the pyramidal layer, and from the frontal primary and secondary motor cortex and the parietal association cortex with separation of superficial (II to IV) and periventricular (V and VI) cortical layers according to Paxinos and Watson. Measurements from the right and left hemispheres were averaged to obtain a single measure.

Stereological methods in the present investigation were thought to be insufficient because of the small study groups.8 Nissl and hematoxylin-eosin stains provided the conventional histological assessment.

**CBF Studies ([14C]IAP Autoradiography)**

In 34 adult male Sprague-Dawley rats (weight, 300 to 350 g), CBF measurements were performed with the [14C]IAP technique.10 Because of the costs, autoradiographic investigations were limited to measurements performed at 2 (n = 5), 6 (n = 6), and 8 weeks (n = 7) after induction of kaolin hydrocephalus. Instead, the control group consisted of 16 animals. This rather large number was needed because CBF measurements are influenced by varying physiological states and environmental factors.10

All the animals were prepared by insertion of polyethylene catheters in both femoral veins and 1 femoral artery. Anesthesia was induced and maintained by spontaneous breathing of 1.5% isoflurane and a 2:1 nitrous oxide/oxygen mixture. After surgical preparation, the rats were immobilized by loose-fitting plaster casts and allowed to recover from anesthesia for 3 hours.10,11 CBF was measured in the awake animals after an intravenous bolus of 20 μCi/300 g [14C]IAP (FA Amersham) diluted with 1 mL of isotonic saline and given at a constant rate (1.2 mL/min) over 50 seconds,10 during which time arterial blood samples were collected. At the end of this period, the animals were killed by an intravenous overdose of barbiturate and immediately decapitated. Brains were rapidly removed, the whole brain specimens were frozen to −75°C in isopentane chilled with liquid nitrogen, and coronal sections of 20-μm thickness were cut.

Sections were exposed on a high-resolution Storage Phosphor screen (Cyclone, Super Resolution Storage Screens, Packard Instruments, Inc; 600×600 dots per inch) for 6 to 7 days. After exposure, the [14C]IAP tissue concentration (microcuries per 100 g) in regions of interest was measured via optical densitometry (digital light units per square millimeter) (Cyclone Imaging System; Packard Instruments, Inc) with the use of a software package provided by the manufacturer (Optiquant; Packard Instruments, Inc). The volume of arterial blood samples was calculated, and the [14C]IAP concentration was determined by liquid scintillation counting.

Local CBF was then calculated (milliliters per 100 g per minute) from the tissue concentration of [14C]IAP, from the blood-brain partition coefficient for the tracer, and from the concentration of the tracer in arterial blood during the infusion period.10 Mean CBF for each region was determined at each of 5 standardized coronal sections throughout the brain. To determine the relationship of ventricular enlargement to CBF, the degree of ventricular dilatation was measured on the coronal autoradiographic sections according to the ratio of the maximum ventricular width divided by the maximum brain width at the level of the anterior commissure, termed the ventricular index.5

**Statistical Analysis**

Immunostaining results and CBF data were analyzed with the aid of a statistical software package (Stat-View) by ANOVA with Fisher’s protected least significant difference (PLSD) for post hoc testing. A probability value <0.05 was regarded as significant.

**Results**

**Immunohistochemical Staining**

**Short-Term Stage**

At 2 weeks, a global increase in NOS immunoreactivity in cortical and hippocampal neurons was observed in the majority of hydrocephalic animals compared with control animals (Figures 1A through 1D and 2A through 2D). Small neurons of the superficial and periventricular cortical layers and of the pyramidal cell layer of the hippocampus displayed...
a homogeneous cytoplasm staining. The quantitative results of NOS staining at various hydrocephalic stages are shown in Table 1.

Hippocampal synaptophysin staining was reduced in the stratum radiatum of the CA1 subfield ($P<0.02$) (Figure 3).

**Intermediate-Term Stage**

At 4 weeks, the most evident finding was an increase in neurofilament staining of the periventricular white matter ($P=0.002$) and the adjacent cortex ($P=0.014$) compared with the more superficial cortical layers, indicating reactive axonal changes (Figure 4). In addition, the number and size of anti-synaptophysin positively labeled puncta in periventricular cortical areas were increased. There was, however, a great variability among animals ($P=0.039$) (Figure 4). Cortical NOS immunoreactivity was already less observed at this stage (Figure 1E and 1F and Table 1).

**Long-Term Stage**

At 6 and at 8 weeks, the periventricular immunoreactivity increases were no longer apparent. However, changes in the hippocampus became more evident. In particular, immunoreactivity of the CA1 subfield was different from changes in the CA3 subfield. In CA1, NOS immunoreactivity in the pyramidal cell layer was maintained during the observed hydrocephalic stages (Figure 2A, 2C, 2E and Table 1). This was followed by decreases in NF68 labeling at 6 and 8 weeks ($P<0.001$) (Figure 3). The pyramidal cell layer showed a loss of staining intensity, and, in the stratum radiatum, there was a loss of the regular radiate arrangement of apical dendrites (Figure 5A and 5B). In contrast, CA3 neurofilament staining increased at 8 weeks ($P<0.001$) (Figure 3). These sections, however, displayed strongly stained varicosities located in the pyramidal cell layer (Figure 5C and 5D).

**TABLE 1. Regional NOS Staining in Kaolin Hydrocephalus**

<table>
<thead>
<tr>
<th>Region</th>
<th>NOS Staining</th>
<th>Controls (n=7)</th>
<th>2 Weeks (n=4)</th>
<th>4 Weeks (n=5)</th>
<th>6 Weeks (n=5)</th>
<th>8 Weeks (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial cortex</td>
<td>Positive</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Periventricular cortex</td>
<td>Positive</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>CA1</td>
<td>Positive</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CA3</td>
<td>Positive</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are the number of positive and negative staining results in the observed regions (positive indicates regular staining of small neurons; negative indicates no staining and/or staining of only isolated neurones). The only samples counted were those that showed at least some cross-reaction with the endothelial isofom in the stains as regularly observed in the control animals. After 2 weeks, a $P<0.01$ (chi-square) for all regions was present. After 4, 6, and 8 weeks, only CA1-staining remained increased ($P<0.01$, chi-square).
Parallel to the neurofilament staining, synaptophysin immunoreactivity also increased in the stratum radiatum of the CA3 subfield, most markedly at 6 weeks ($P_{/H11021}=0.02$) (Figure 3). The regular punctate pattern of synaptophysin staining, located in the apical dendritic layer of the pyramidal cells, was markedly increased (Figure 5G and 5H). In the CA1 sector, however, no significant reactive increases of synaptophysin immunoreactivity were seen, and staining was chronically reduced (Figure 3). Again, anatomic disarrangement in the staining pattern of both the pyramidal layer and the stratum radiatum was apparent (Figure 5E and 5F).

In the cortex, the most relevant findings were obtained with neurofilament protein staining (Figure 4). In the superficial pyramidal layers, there was a shift from the regular dendritic staining toward a strong and an increased selective labeling of neuronal perikarya. No significant morphological changes in the Nissl and hematoxylin-eosin stains were observed during the hydrocephalic stages. No chromatolysis or pyknosis was found in either the hippocampal pyramidal cell layer or the cortex.

**CBF Measurement**

The mean values for local CBF for all regions at the various hydrocephalic stages are shown in Table 2. At 2 weeks, a global reduction in blood flow was found, with levels that were markedly reduced compared with control animals ($P_{/H11021}<0.001$, Fisher’s PLSD). At 6 and 8 weeks, blood flow was nearing normal, although regional differences in CBF reduction, compared with the controls, were still observed.

**Short-Term Stage**

At 2 weeks, reductions in CBF in the cortical gray matter were more marked in periventricular than in superficial layers, with an average decrease of $18\pm4\%$ (mean$\pm$SEM) in the cortical gray matter.
the periventricular cortex and 13±4% in the superficial cortical layers. However, maximum decreases of approximately 50% in both periventricular and superficial cortical layers were observed. In the hippocampus, CBF was reduced by 23±5%, with a maximum reduction of 55% observed. No differences between the CA1 and CA3 subfields were found. The largest decreases in CBF were found in the periventricular white matter, with an average reduction of 53±4%, up to a maximum of approximately 70%.

**Long-Term Stage**

At 6 weeks, average blood flow values in superficial cortical layers returned to control levels. In the periventricular layers, blood flow was still significantly reduced, although only by 15±3% compared with controls. Maximum CBF reductions in the superficial cortex did not exceed 40% and in the periventricular cortex did not exceed 50%. In the hippocampus, the average CBF was not significantly reduced compared with controls (13±3%), but maximum decreases of CBF up to 50% were still observed. In the white matter, however, average CBF was decreased by 51±7%, and maximum reductions reached a striking 85%.

At 8 weeks, average CBF was normalized in both cortical and hippocampal regions. Maximum decreases did not exceed 20% in the cortex and 25% in the hippocampus. Striking CBF reductions were seen only in the periventricular white matter. These reductions, however, were less marked than at 6 weeks (33±6%). Still, a maximum reduction of up to 60% was seen.

**CBF and Ventricular Enlargement**

At 2 weeks, the ventricular index was increased compared with controls (0.4±0.05 versus 0.33±0.05; *P*=0.003). Maximum dilatation occurred at 6 weeks, with a ventricular index of 0.55±0.05 (*P*<0.0001). Ventricular width decreased at 8 weeks compared with animals in the 6-week group (0.45±0.1 versus 0.55±0.05; *P*=0.002).

In Figure 6, the sequential changes in local CBF for both the cortex and the hippocampus are compared with the changes in ventricular index. The time courses demonstrate no correlation between ventricular enlargement and decreased CBF. At the stage of maximum ventricular index, blood flow was already recovering. Except for a low correlation in the periventricular white matter (*R*²=0.5; *P*<0.0001), regression analysis of blood flow values plotted against the ventricular index in all animals showed no significant relationship. This holds for all observed regions at all stages of hydrocephalus.

**Discussion**

In this adult model of hydrocephalus, the cisternal injection of kaolin causes an inflammatory reaction and results in a progressive, but variable, dilation of the ventricles. As chronic hydrocephalus develops, stabilization of ventricular enlargement and normalization of intracranial pressure occurs, in part because of the opening of compensating alternative cerebrospinal fluid pathways. This model therefore may be somewhat similar to some forms of chronic hydrocephalus seen in humans, eg, posthemorrhagic and postmeningitic hydrocephalus, as well as to the poorly understood syndrome of normal pressure hydrocephalus.

The immunochemical markers were chosen to demonstrate neuronal damage that was not apparent on Nissl-stained histological sections. The selected markers reflect enzymatic, toxic, and cytoskeletal neuronal responses. The aim was to investigate immediate and chronic, reactive changes. Neuronal NOS is primarily expressed in selective neuronal populations of the central and peripheral nervous system, and its activity is known to play a major role in mediating acute excitotoxic damage, eg, after cerebral ischemia. Among the 3 neurofilament subtypes, NF68 is most sensitive to both

**Figure 6.** Changes of the ventricular index (VI) measured at the anterior commissural level compared with changes in local CBF of both the hippocampus and the cortex at 2 (H2), 6 (H6), and 8 (H8) weeks after hydrocephalus induction and in controls (C). Values are mean±SE. *P*<0.01 for both cortical and hippocampal CBF compared with the control level (Fisher’s PLSD). The ventricular index showed *P*<0.01 for all observed weeks.
cytoskeletal injury and the onset of reactive dendritic and axonal changes. Synaptophysin (38 kDa) has been identified as a specific component of the membrane of presynaptic vesicles and is thought to be of major importance in hippocampal neuromodulation and in synaptic plasticity.

In the present investigation local changes in CBF, as measured quantitatively by $[^{14}C]$IAP autoradiography, were compared with neuronal responses, as demonstrated by immunohistochemistry, during the course of kaolin-induced hydrocephalus. Both the CBF measurements and the neuronal responses displayed significant regional and temporal alterations that were correlated in both the short-term and long-term hydrocephalus stages.

**Immunohistochemical Profiles**
The observed global immunohistochemical neuronal reaction seen in the short-term stage, as demonstrated by the cortical and hippocampal increase in NOS staining, appear to indicate a toxic neuronal response. NOS activation has been reported to be secondary to an elevation of extracellular glutamate.

The present observations are in agreement with the findings of others who found an increase in extracellular glutamate transporters in the short-term period after the induction of kaolin hydrocephalus. Immunoreactivity in the intermediate stage displayed a periventricular "reactive" profile, suggestive of physical stretching and subsequent axonal derangement, that correlated with the enlargement of cerebral ventricles.

The long-term stage, however, was characterized by ongoing immunohistochemical changes in areas known to be vulnerable to ischemia, eg, the pyramidal layer of the CA1 subfield of the hippocampus. The decreased neurofilament reactivity is indicative of neuronal degeneration. Together with the chronic reduction of synaptophysin reactivity seen, the response in the CA1 subfield resembles findings of "delayed neuronal death," a phenomenon that has been regularly observed after transient global ischemia. Because the maintained NOS reactivity precedes the neurofilament degradation, toxicity to the CA1 neurons due to free radical activation is also suggested.

The neuronal response in the CA3 subfield, however, is suggestive of cellular recovery and adaptive processes. The pattern of focal increased neurofilament reactivity, located at the perikarya of the pyramidal cells, resembles similar findings seen after experimental traumatic brain injury and could be attributed to axonal regeneration. Furthermore, the increased synaptophysin staining demonstrates both plasticity and synaptic recovery and may correspond to the establishment of multiple synaptic contacts with the proximal portions of pyramidal cell apical dendrites.

**Hippocampal Response in Kaolin-Induced Hydrocephalus**
Very few studies have described damage to the hippocampal formation in adult hydrocephalus. In particular, experimental hydrocephalus in the immature animal has evidenced involvement of the hippocampus. Recently, a study of Schaffer collateral responses in the CA1 pyramidal cell layer suggested disturbances in long-term potentiation of population spikes in adult kaolin-induced hydrocephalus.

**CBF Changes in Kaolin-Induced Hydrocephalus**
Quantitative autoradiographic studies on CBF in experimental kaolin-induced hydrocephalus have only rarely been performed and, when reported, are in the neonatal model. These studies have all suggested significant CBF decreases in the early stages of the disease. Our study has shown significant reductions of CBF in the short-term and intermediate-term stages, although blood flow was virtually restored at 8 weeks. It could be argued that the reduction in CBF that is seen in kaolin-induced hydrocephalus is due to vascular narrowing secondary to the meningeal inflammatory response, but we found no histological evidence to support that interpretation.

In regard to the mechanism of CBF reduction in kaolin-induced hydrocephalus, our findings did not suggest that the blood flow alterations were a direct result of ventricular enlargement. Blood flow levels started to return to normal while the ventricles still progressed. Although intracranial pressure was not measured in these rats, data from previous studies indicate that the initial intracranial pressure elevation after hydrocephalus induction would be sufficient to cause cerebrovascular compression resulting in the CBF decrease seen during short-term hydrocephalus stages in the present study.

On the basis of the existing knowledge of ischemic thresholds, an impairment of protein synthesis and disturbed energy metabolism would imply reductions of CBF far beyond 50%. Neither cortical nor hippocampal blood flow levels were below this threshold in the present study, indicating that blood flow impairment in the time course of kaolin-induced hydrocephalus is only moderate and might not be expected to account for the neuronal dysfunction seen in the long-term stage. Only white matter flow was found to be below the ischemic threshold, which corresponds to recent findings of calcium-mediated proteolytic damage in cerebral white matter of immature and adult kaolin-induced hydrocephalus.

**CBF Changes and Neuronal Responses**
The toxic neuronal response in the short-term hydrocephalic stages can be correlated with the significant global blood flow reduction that precedes ventricular enlargement. However, what causes the selective hippocampal injury seen in the long-term stage? At that time, CBF has returned to nearly normal values, and ventricular size is also decreasing.

In a model incorporating an arteriovenous fistula that chronically reduced global CBF in rats by approximately 25% to 50%, it has recently been demonstrated that these "nonischemic" reductions of CBF induce a delayed selective neuronal injury. The authors found a disturbed long-term potentiation in CA1 neurons of the hippocampus and successfully demonstrated subtle morphological changes in the CA1 subfield using light microscopy and transmission electron microscopy.

Given that there are differences in the pathology of arteriovenous fistula– and kaolin-induced hydrocephalus, our
observations on the neuronal response in the long-term stage of kaolin hydrocephalus strongly resembles the findings of arteriovenous fistula–induced ischemia. We postulate that the moderate to mild but prolonged reductions of CBF in kaolin-induced hydrocephalus may cause the delayed selective neuronal injury evidenced by the immunohistochemical changes that we saw in both the CA1 and CA3 subfields. Corresponding to our findings, the role of cerebrovascular mechanisms in the pathophysiology of chronic hydrocephalus has recently been demonstrated as well, suggesting that cerebrovascular adaptive processes allow maintenance of adequate cerebral perfusion and metabolic support in the hypoxic environment of chronic hydrocephalus.

In conclusion, in the present descriptive study, we showed a correlation of both the short-term and the long-term neuronal responses with a moderate but prolonged reduction of CBF that was demonstrated during the first weeks after hydrocephalus induction. Because it indicates significant immunohistochemical evidence of neuronal injury caused early in the course of induced hydrocephalus, the present study might have important clinical implications for early shunting. Physicians tend to be conservative to avoid the surgical risks associated with shunting, as well as the risk of persistent shunt dependency. Withholding shunt treatment might have serious neuropsychological sequelae for the patient.

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