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The Effect of Intravascular Saline Perfusion on the Sequelae of Transient Cerebral Ischemia

Light and Electron Microscopial Observations

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Summary. Transient global ischemia was produced in cats by interrupting the arterial blood supply to the brain under direct observation of the pial vessels. The pial circulation could be restored only for a brief period after ischemia but intravascular rinsing of the brain during ischemia with various saline solutions considerably improved the postischemic circulation.

The functional status of neuronal activity was assessed by recording the EEG and the pyramidal response (PR) after electrical stimulation of the motor cortex. Perfused and nonperfused cats were compared with regard to the structure and function of the motor cortex in the early postischemic period. The neurophysiological signals recovered after ischemia of much longer duration in the perfused animals than in the nonperfused cats. Severe structural alterations were seen in capillaries, neurons and glial cells when ischemia was long enough to suppress the PR. In the perfused animals these changes were virtually absent even after ischemia up to 30 min duration.

The increased tolerance of the brain to ischemia produced by the intravascular rinsing appears to result from at least two different mechanisms. Elimination of metabolic waste products presumably reduces tissue damage during ischemia and the improved postischemic circulation prevents secondary ischemic lesions.

Zusammenfassung. Passagere Globalischämie wurde an Katzen durch Unterbrechung der Blutversorgung des Gehirns unter direkter Beobachtung der Piagefäße erzeugt. Die piale Zirkulation konnte nur kurzfristig nach der Ischämie wiederhergestellt werden, doch wurde die postischämische Zirkulation durch intravasale Perfusion des Gehirns mittels verschiedener Salzlösungen beträchtlich verbessert.

Der Funktionszustand der neuronalen Aktivität wurde mittels EEG und Pyramidenreaktion (PR) nach elektrischer Reizung des motorischen Cortex geprüft. Perfundierte und nichtperfundierte Katzen wurden im Hinblick auf die Struktur und Funktion des motorischen Cortex in der frühen postischämischen Periode verglichen. Bei perfundierten Tieren kamen die neurophysiologischen Signale nach Ischämie von wesentlich längerer Dauer wieder, als bei nichtperfundierten Katzen. Schwere strukturelle Veränderungen fanden sich an Capillaren, Neuronen und Gliazellen, wenn die Ischämie lange genug anhielt, um die PR zu unterdrücken. Bei perfundierten Tieren fehlten solche Veränderungen selbst nach Ischämie bis zu 30 min Dauer.

Die gesteigerte Toleranz des Gehirns gegenüber Ischämie infolge intravasaler Durchströmung erscheint durch zumindest zwei verschiedene Mechanismen bedingt. Die Eliminierung metabolischer Schlackenstoffe verringert vermutlich die Gewebsschäden während der Ischämie und die verbesserte postischämische Zirkulation verhindert sekundäre ischämische Schäden.

Key-Words: Transient Cerebral Ischemia — Pial Circulation — Neurophysiology — Electron Microscopy.

Introduction

Brain damage caused by transient circulatory arrest is probably not the effect of oxygen and glucose deprivation alone. Tissue acidosis and accumulated catabolic products may play a significant role in the genesis of permanent ischemic brain damage (Lindenberg 1956, 1963; Friede and van Houten; Bakay and Lee). Transient interruption of the cerebral blood flow may also be complicated by an impaired intracerebral circulation after ischemia (Courville; Kabat *et al.*; Ames III *et al.*; Chiang *et al.*). Electron microscopical data indicate that swelling of the endothelial and the perivascular glial cells may occur to such an extent during ischemia that the lumen of the intracerebral capillaries will be reduced even to a fine slit (Chiang *et al.*). This swelling combined with increased viscosity of the blood and postischemic hypotension are probably the most important factors causing this so-called "no-reflow phenomenon" (Kowada *et al.*).

To analyze the role of various factors which can influence the central effects of transient ischemia, a model is required by which acute ischemia can be produced without impaired postischemic circulation and in which the subsequent functional and morphological changes can be recorded. Acute ischemia can be produced in the cat by clamping all major arteries supplying the brain and the circulation after ischemia can be improved by rinsing the cerebral vessels with saline solutions during ischemia (Hossmann and Olsson). A convenient way to follow the functional sequelae is the recording of the electroencephalogram (EEG) and of the pyramidal response (PR). Quantitation can then be used in describing the functional impact of ischemia and the recovery thereafter.

Our previous study revealed that the saline perfusion considerably improved the recovery process after ischemia (Hossmann and Olsson). Both the EEG and the PR reappeared after transient circulatory arrest of much longer duration than could be tolerated in nonperfused cats. The present report concerns light and electron microscopical observations in cats subjected to transient cerebral ischemia with or without rinsing of intracerebral vessels during ischemia.

Material and Methods

42 cats were anesthetized with sodium pentobarbital (30 mg/kg, Nembutal), immobilized with gallamine triethiodide (10 mg/kg, Flaxedil) and artifically respirated. Global cerebral ischemia was produced by temporary clamping of the major arteries supplying the brain for various periods of time i.e. subclavian und innominate arteries close to aorta or both carotid and basilary arteries. The basilary artery was ligated from the ventral side through an opening in the occipital bone. Other detail abouts the preparation of the cats have been presented elsewhere (Hossmann and Olsson).

The efficiency of the clamping in producing ischemia was controlled by direct observations of the pial circulation through a craniotomy over the right suprasylvian gyrus. The experiments were classified into two major categories with regard to the circulation after ischemia (cf. Hossmann and Olsson). In brains with "unimpaired recirculation" a noncorpuscular blood flow could be observed in all pial vessels under a dissection microscope and there was an almost immediate appearance of Evans blue that had been injected intravenously. In such cats the fixation by perfusion resulted in a uniform hardening of the brain and red blood cells were washed out from almost all vessels by the fixative. Brains which did not fulfill these criteria were considered "inproperly recirculated".

We have previously observed that with the present model global ischemia of more than 8 min duration is followed by an impaired recirculation which becomes apparent 20 min or later after release of the arterial clamps (Hossmann and Olsson). Group 1 therefore comprises

12 cats with ischemia of 8—90 min duration and a survival time between 20 min and 4 h. However, if the initial ischemic period is shorter or if the cats are taken within 20 min, there are no signs of impaired circulation in the pial vessels. Group 2 comprises 18 cats with 1 to 90 min of initial ischemia and 5 min to 3 h survival. We have also found that rinsing of the cerebral vessels during ischemia with various saline solutions eliminates the signs of "no-reflow" (Hossmann and Olsson). Group 3 consists of 12 cats with intravascular rinsing during ischemia of 14—30 min duration and up to 3 h survival. Ischemia was produced in these cats by clamping the subclavian and the innominate arteries. The solutions (Ringer or calf serum or 6% dextran) were infused through a catheter placed above the other ligations in the right carotid artery (pressure 50—80 mm Hg, flow rate 10—20 ml per min, room temperature). The solutions were allowed to pass into the general circulation after the passage through the brain.

Neurophysiological Methods. The EEG was recorded by bipolar silverball electrodes from the gyrus suprasylvius. The pyramidal response (PR) was recorded after stimulation of the right sensorimotor cortex by bipolar concentric electrodes placed stereotactically into the pyramidal tracts at the lower level of the pons and displayed on a Tectronix 502 oscilloscope. The PR is composed ot two major components (Patton and Amassian): the first "direct" response (D-wave) is due to the electrical stimulation of the motoneurons and the pyramidal fibers. The following "indirect" response (I-wave) is elicited by the motoneurons which have been synaptically activated by interneurons in the motorcortex. The functional state of the motorcortex is reflected by the form and the amplitude of the two waves, the suppression of the indirect response indicates that the motoneurons can no longer be activated synaptically. The suppression of the direct response indicates that the motoneurons or their axons can no longer be activated electrically. The PR is more resistant to various lesions than the EEG. It is particularly suited for recording of severe functional deficits and for detecting early signs of recovery after ischemia.

The physiological findings have been presented elsewhere (Hossmann and Olsson). Briefly, there was no difference in the suppression times of the EEG (20 ± 5 sec) or of the PR (I-wave 4 ± 2 min, D-wave 6 ± 2 min) between cats subjected to ischemia with or without vascular rinsing. However, considerable differences were recorded after the transient ischemic period. The D-wave of the PR recovered only in some of the nonperfused animals (within 40 min after ischemia of less than 16 min duration) and when ischemia lasted more than 8 min the recovery process was interrupted and reversed. This secondary suppression coincided in time with the onset of the impaired postischemic pial circulation. In the perfused animals, the D-wave consistently recovered within 10 min after ischemia lasting as long as 30 min and no secondary suppression was recorded.

Morphological Methods. The brains from group 2 and 3 were fixed by perfusion through the aorta or through the carotid artery. From group 1 cortical biopsies were fixed by immersion, since adequate fixation by perfusion can not be obtained.

The perfusion was started with McEwen's solution and fixation was accomplished by a sodium cacodylate buffered mixture of purified glutaraldehyde and paraformaldehyde at pH 7.1—7.2 (Karnovsky). After rinsing in cacodylate buffer, pieces from the cortex were postfixed in osmium tetroxide, dehydrated in methanol and embedded in Araldite. One micron thick sections were cut on a Porter-Blum microtome and stained with toluidine blue for light microscopy. The thin sections for electron microscopy were stained with lead citrate (Venable and Coggeshall). Other blocks were paraffin embedded and sections were stained with various routine methods.

Results

Ischemia without Rinsing of Intracerebral Vessels. Except for the severity of the changes in the endothelial and the perivascular glial cells the lesions were similar in animals of group 1 and 2. The results will therefore be described together:

Light microscopical changes were consistently present in the cerebral cortex when ischemia lasted more than 8 min, and the animals had survived longer than 30 min. In such animals several shrunken dark staining neurons were seen in the

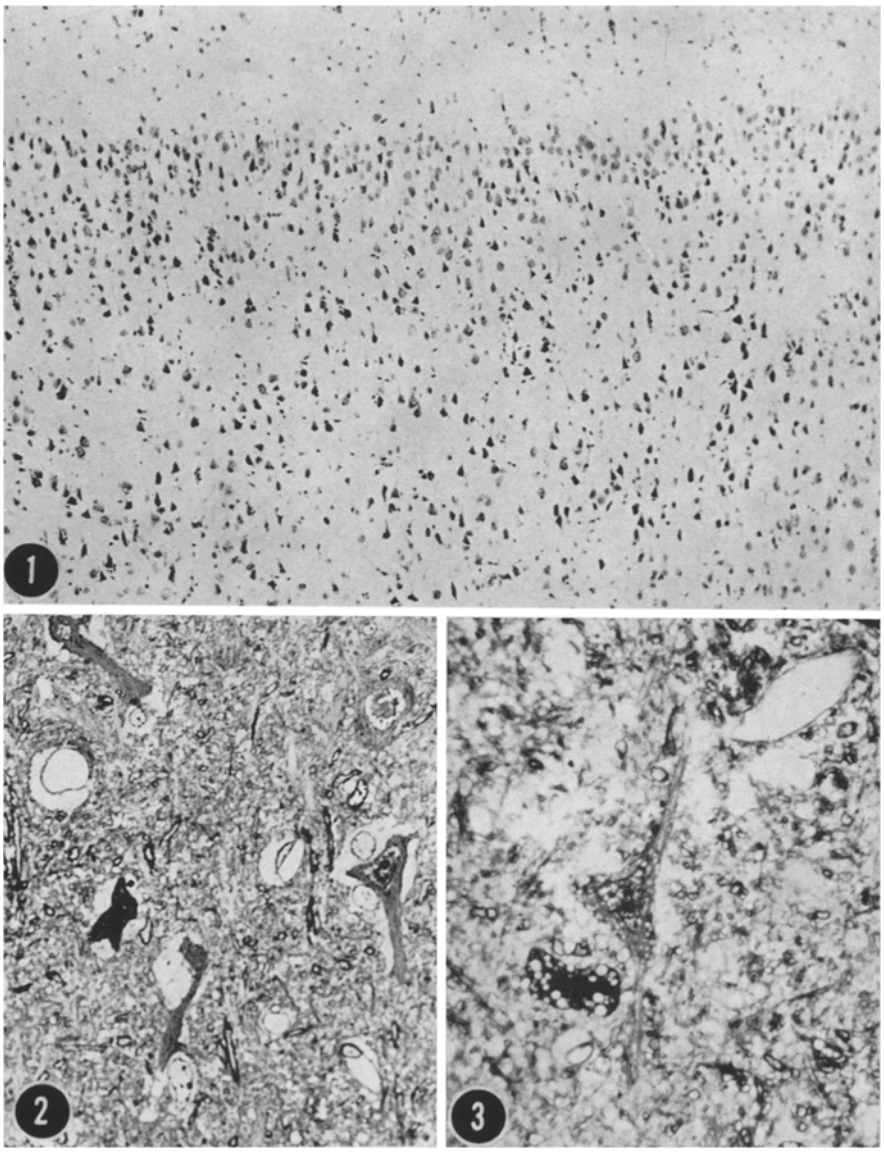


Fig. 1. Numerous shrunken dark neurons in the cerebral cortex following ischemia without intravascular perfusion. Paraffin section. Cresyl violet

Fig. 2. Dark neurons surrounded by clear cell processes and swelling of perivascular glial cells in a noninfused cat subjected to ischemia. Araldite section. Toluidine blue

Fig. 3. Vacuolization of neuronal cytoplasm following ischemia without vascular perfusion. Araldite section. Toluidine blue

paraffin embedded material (Fig. 1) but incrustations of the Golgi network were only occasionally encountered. The changes were more easily seen in the plastic embedded toluidine blue stained sections e.g. irregular form and poor staining

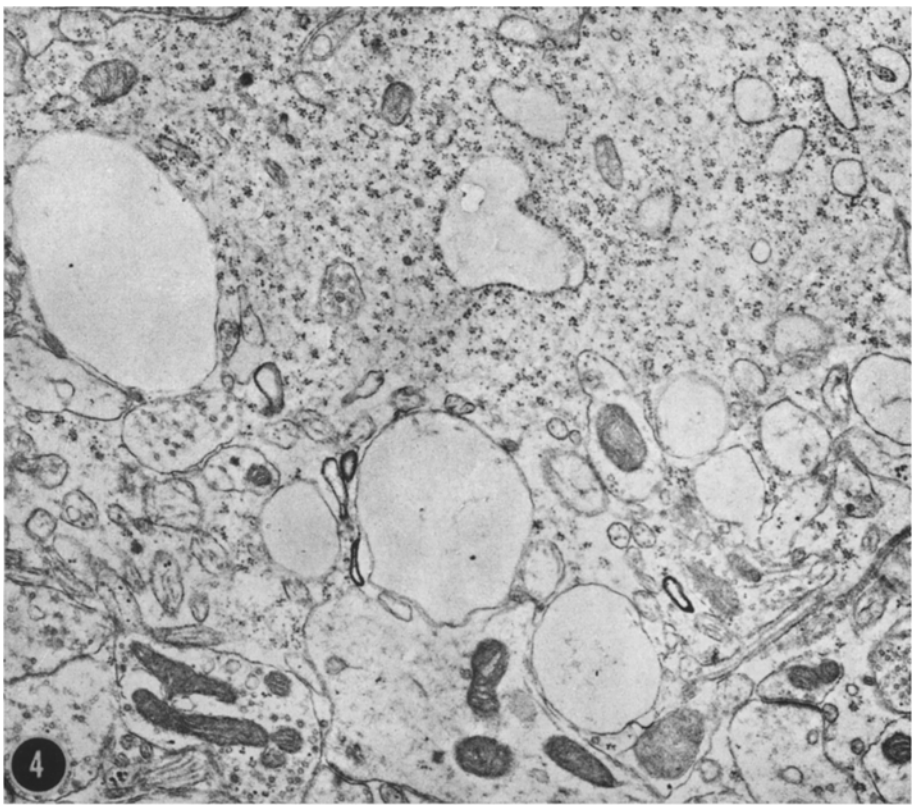


Fig. 4. Severe swelling of organelles, presumably part of endoplastic reticulum. Specimen from cerebral cortex of a cat subjected to ischemia without intravascular perfusion during ischemia

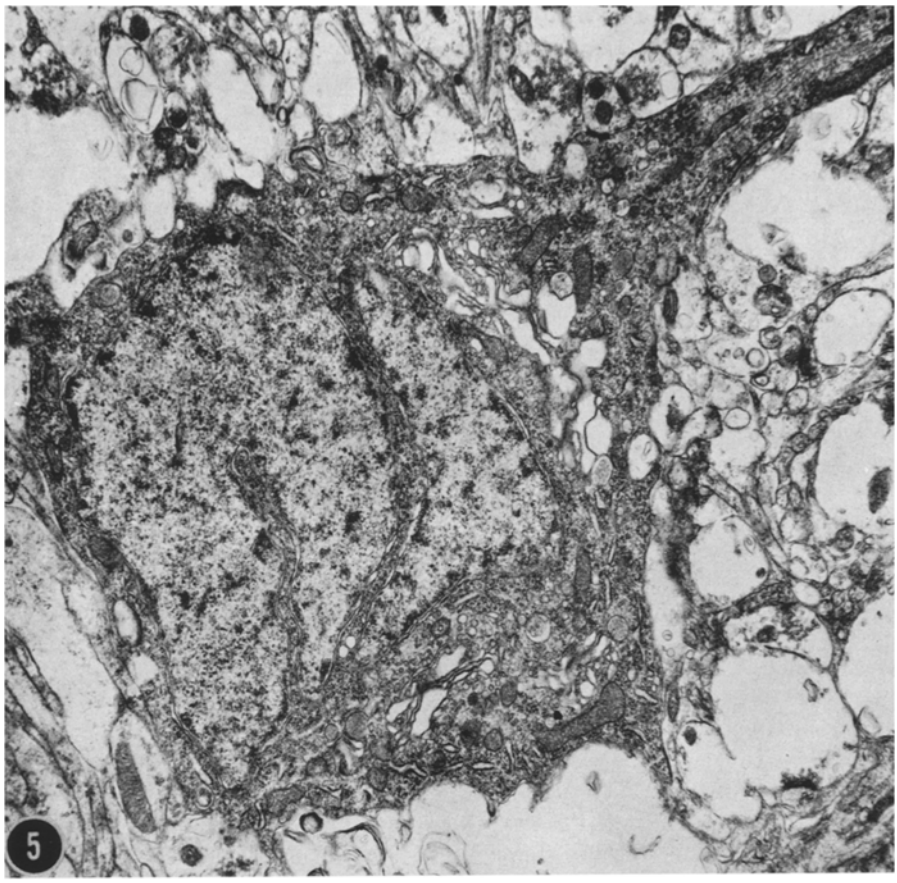
of nuclei, cytoplasmic vacuolization, dark neurons and neurons with generalized poor staining of soma and processes (Figs. 2 and 3).

The fine structural changes in the neurons which appeared poorly stained by light microscopy were similar to those first described by Hager *et al.* in the Syrian hamster following hypoxia. Both the nuclei and the cytoplasm were swollen, the nuclear and cytoplasmic membranes were irregular and occasionally broken. Severe mitochondrial changes were also observed including excessive swelling, fragmentation and disorganization of cristae and reduced electron density of the matrix. Parts of the endoplasmic reticulum and cisternae of the Golgi complex were also abnormally wide (Fig. 4).

Electron microscopically the dark neurons were easily recognized by their form and electron density of the cytoplasm (Figs. 5 and 6). These cells were fre-

Fig. 5. Dark neuron in the cerebral cortex following ischemia without artificial perfusion. Note the shrinkage of the cells and the characteristic identations of surrounding cellular processes

Fig. 6. Part of the cytoplasm of a dark neuron showing mitochondrial changes and densely aggregated organelles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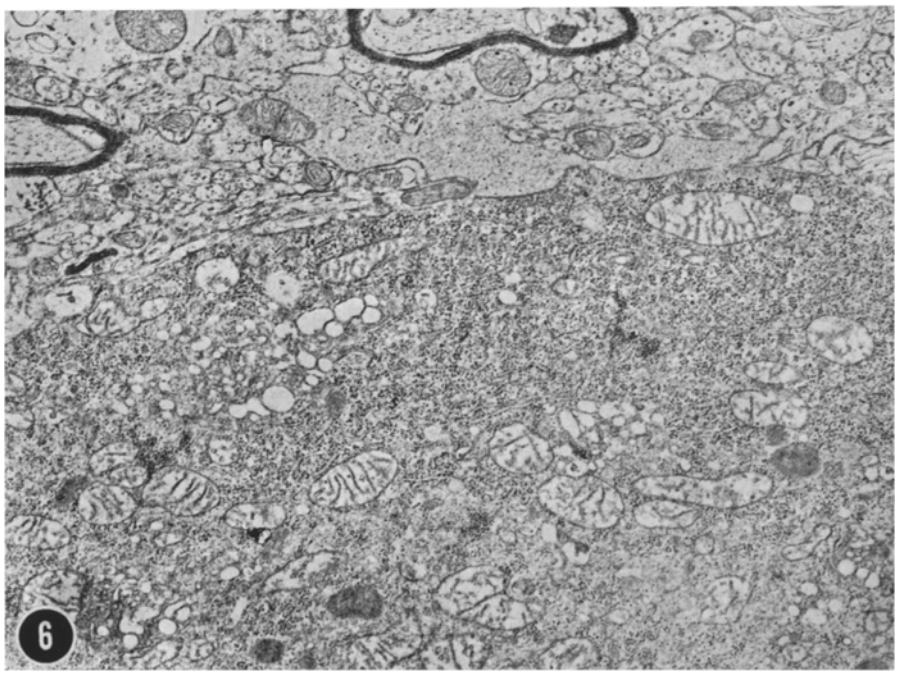
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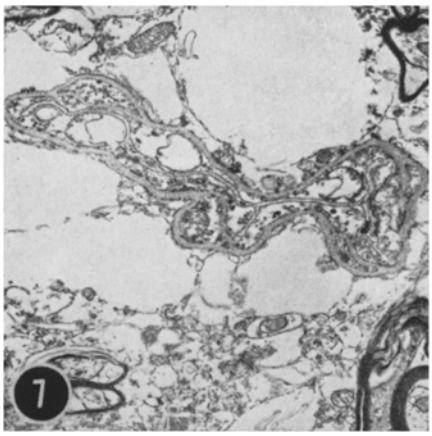


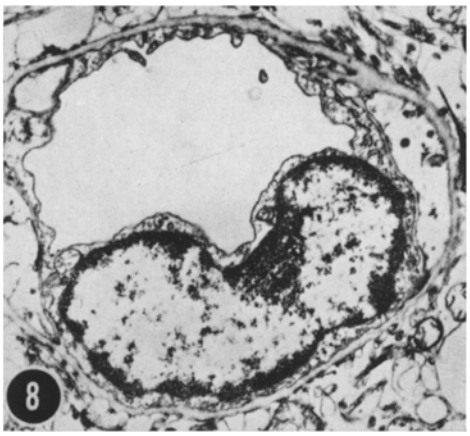


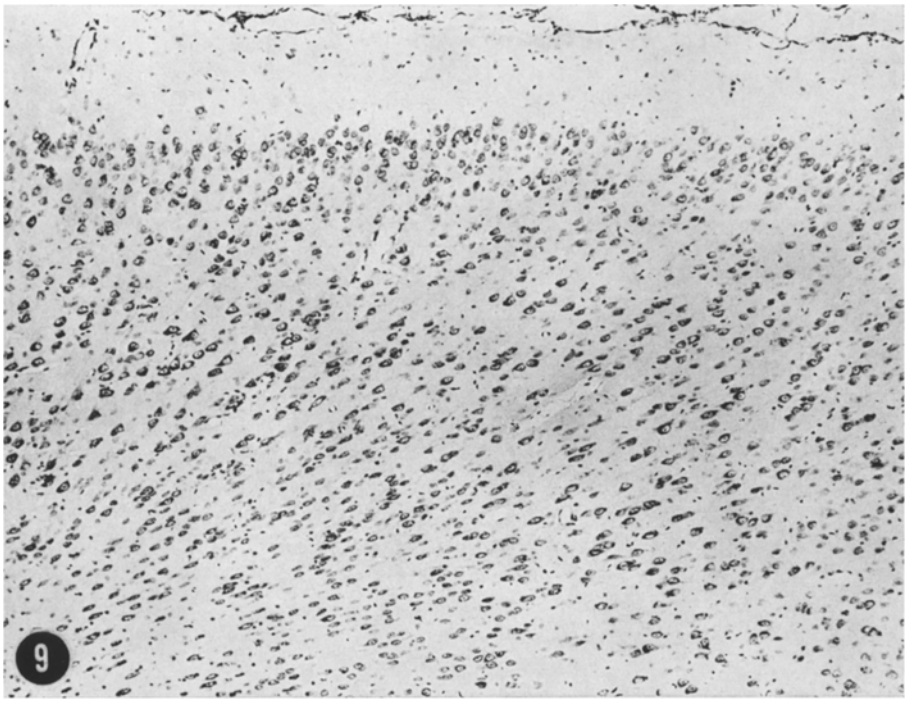
Fig. 7. Swelling of perivascular glial cells after ischemia without artificial perfusion. Note also the endothelial swelling and the severe reduction of the size of the lumen

Fig. 8. Intracerebral vessel shortly after transient cerebral ischemia without artificial perfusion. Note the patency of the lumen

quently surrounded by swollen cell processes which characteristically bulged into the cytoplasm of the neuron. The organelles were densely aggregated in the cytoplasm. In many of these cells the mitochondria were swollen, their cristae broken and the matrix appeared clear. There was also occasionally abnormal dilatated parts of the Golgi complex. The endoplasmic reticulum appeared normal or slightly dilatated and the ribosomes were closely aggregated (Fig. 6).

The patency of the arteries supplying the brain from the aorta to the circulus of Willis was ascertained post-mortem by demonstrating free flow of fluids infused into the heart. No signs of thrombotic or embolic occlusion could be observed in the intracerebral vessels. However, the perivascular glial cells were considerably swollen in animals with signs of impaired circulation (group 2) (Fig. 7). In such animals, the lumen was severely obliterated and it often remained only as a fine slit. Intravascular "blebs" as observed by Chiang *et al.* were only rarely encountered but many endothelial cells were swollen and showed mitochondrial changes. In some areas the plasma membrane of the endothelial cells was broken but the junctions between adjacent endothelial cells appeared intact. The severity of the blood vessel changes appeared to be directly related to the status of the pial circulation after ischemia: fewer and milder structural alterations in cats without impaired pial circulation (group 2) (Fig. 8) and the most severe alterations in cats with impaired pial blood flow (group 1).

Ischemia with Rinsing of Intracerebral Vessels. The morphological appearance of the cerebral cortex after rinsing the cerebral vessels during ischemia was quite different from that in the preceding groups. Light microscopically wide areas of the cortex appeared entirely normal both with regard to the neuronal distribution and morphology (Fig. 9). However, in small parts of the cortex shrunken dark neurons were observed. They were absent or very rare when the survival time was short (1—15 min) but more frequent in animals with signs of functional recovery and long survival time (30 min to 3 h).



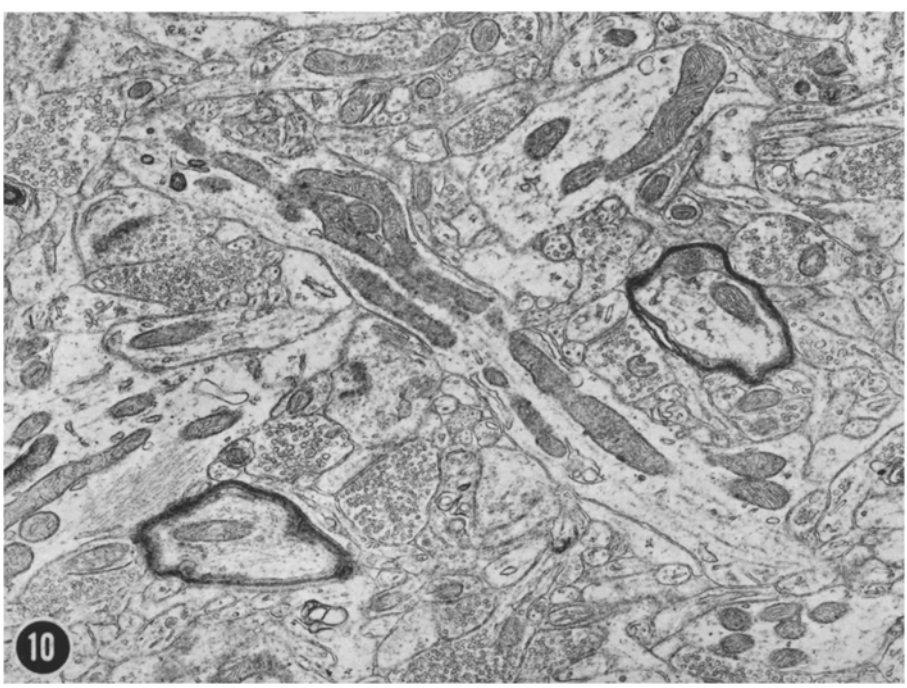
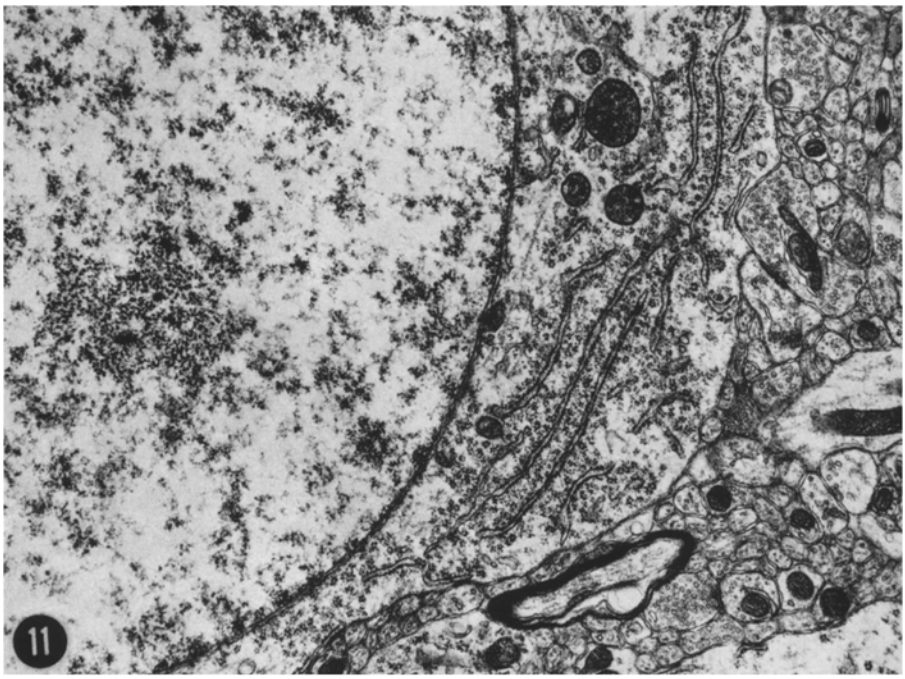


Fig. 9. Cerebral cortex from a cat subjected to ischemia combined with Ringer perfusion during ischemia. Note the normal cytoarchitectonic and the absence of cellular changes. Cresyl violet

Fig. 10. Part of the neuropil with normal appearance following ischemia combined with Ringer perfusion



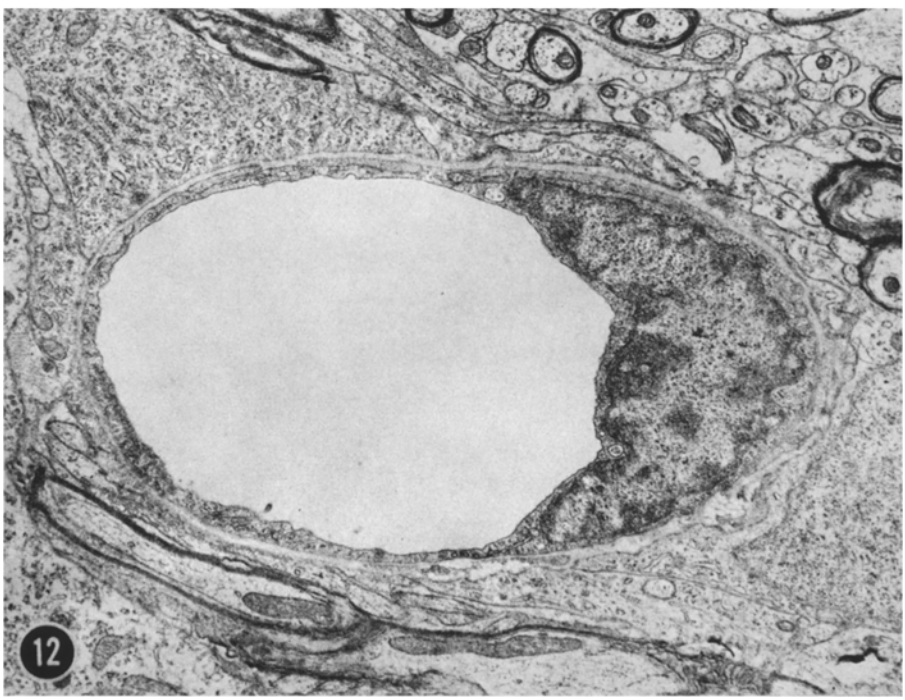


Fig. 11. Part of a neuron in the motor cortex following ischemia combined with Ringer perfusion. Note the absence of intracellular changes

Fig. 12. Small intracerebral vessel with normal appearance following ischemia combined with Ringer perfusion

Electron microscopy confirmed the light microscopical findings of a remarkable absence of alterations in cortical neurons (Figs. 10 and 11). The nuclei, the cytoplasmic organelles, the plasma membrane and the synapses were usually entirely normal. The fine structure of the dark neurons was similar to that described above. In the neuropil we occasionally observed a slight swelling of cellular processes sometimes accompanied by distortion or fragmentation of cellular membranes.

The glial cells were normal in most of the animals. In cats surviving more than 2 h the number and the position of satellite cell around neurons suggested that a proliferation of such cells had taken place. No abnormal swelling of perivascular astrocytes or of endothelial cells was seen and there was no formation of intravascular "blebs" (Fig. 12).

Discussion

Recent experimental studies on transient global cerebral ischemia have revealed that the return of blood into the areas afflicted by the ischemia is severily impaired (Ames III *et al.*; Kowada *et al.*; Chiang *et al.*). This so-called "no-reflow phenomenon may be apparent immediately after ischemia of a duration as short as 5 min and involves more than 50% of the brain volume within 15 min (Cantu and Ames III). Occlusive changes in the terminal blood vessels caused by swollen endothelial and perivascular glial cells, postischemic hypotension and increased viscosity of the blood are the most important pathogenetic factors for this condition (cf. Ames III *et al.*). This phenomenon may be of particular importance in causing permanent brain injury after transient ischemia since the return of blood is impaired and consequently the possibility of recovery eliminated.

We have previously observed that a slow rinsing of the cerebral vessels during transient ischemia with various saline solutions considerably improves the pial circulation after ischemia (Hossmann and Olsson). The present study revealed that cats subjected to transient ischemia combined with such rinsing do not show the occlusive vascular changes which otherwise are present. Since the vasculatory changes induced by ischemia may be influenced by our procedure it would be important to reveal the cause of the cellular swelling, which appear to play a crucial role in the manifestation of the "no-reflow" phenomenon. Chiang *et al.* were of the opinion that the progressive anoxia and loss of substrates during ischemia caused failure of active sodium transport out of the brain cells leading to a movement of electrolytes and water from the extracellular to the intracellular compartment. Our observations indicate that anoxia is not the only factor in ischemia causing cellular swelling. Accumulation of catabolic products and/or changes in the pH of the tissue may also have profound effects (Friede and van Houten).

The significance of the vascular perfusion for the outcome of transient ischemia is evident when we compare nonperfused and perfused cats in the postischemic period with regard to the structure and function of the cerebral cortex. Brown and Brierley have previously studied the early stages of anoxic-ischemic nerve cell damage by light microscopy. The first appearing alteration consisted in microvacuolation of the cytoplasm which could be detected as soon as 40 min after the onset of their experiments. They considered that these vacuoles were caused by

distended mitochondria. Distended parts of the Golgi complex and of the endoplasmic reticulum may also contribute to the light microscopical appearance. The further sequence of cell changes in Brown's and Brierley's experiments was ischemic nerve cell degeneration within 24 h, later degeneration with incrustations, and finally cell loss. In our material a large number of dark neurons were present and incrustated neurons were virtually absent. The ultrastructural changes in ischemic cats without vascular rinsing closely resembled what has previously repeatedly been described in various ischemic or anoxic conditions of the central nervous system (cf. Hager *et al.*; Hills; Webster and Ames III). In the neurons there is an early swelling of the mitochondria, followed by progressive swelling of Golgi complex and of the endoplasmic reticulum (Webster and Ames III). Such changes may be reversible, but later irreversible discontinuities of organelles and cellular membranes become visible.

We have previously observed that perfused animals show signs of neuronal recovery (reappearance of the PR or of the EEG) after ischemia of longer duration than can be tolerated by nonperfused animals. The results of this investigation show that these animals almost entirely lack the morphological changes typical for early ischemic lesions, thus providing a reasonable structural correlate to the functional difference previously recorded.

The mechanisms by which the vascular perfusion reduce the ischemic brain damage are not yet fully understood. However, the improvement of the postischemic circulation induced by the vascular rinsing certainly is a factor of major importance since it provides the possibility of recovery. Factors such as the lowering of the cerebral temperature and the effects of remaining oxygen in the perfusate are probably of minor importance, since only a small volume of fluid is used and cooling of the perfusate was avoided. Instead, it is reasonable to assume that a major effect of the perfusion is to interfere with the accumulation of metabolic waste products. In this connection it should be recalled that lactic acid formed by anaerobic glycolysis has been considered by several authors as being of particular importance for the structural alterations occuring during and after ischemia (Lindenberg 1956, 1963; Bakay and Lee)

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References

Ames III, A., Wright, R. L., Kowada, M., Thurstone, J. M., Majno, G.: Cerebral ischemia. II. The No-reflow phenomenon. Amer. J. Path. **52**, 437—454 (1968).

Bakay, L., Lee, J. C.: The effect of acute hypoxia and hypercapnia on the ultrastructure of the central nervous system. Brain **91**, 697—706 (1968).

Brown, A. W., Brierley, J. B.: The nature, distribution and earliest stages of anoxic-ischemic nerve cell damage in the rat brain as defined by the optical microscope. Brit. J. exp. Path. **49**, 87—106 (1968).

Cantu, R. C., Ames III, A.: Distribution of vascular lesions caused by cerebral ischemia. Neurology (Minneap.) **19**, 128—131 (1968).

Chiang, J., Kowada, M., Ames III, A., Wright, R. L., Majno, G.: Cerebral ischemia. III. Vascular changes. Amer. J. Path. **52**, 455—476 (1968).

Courville, C. B.: Cerebral anoxia. Los Angeles: San Lucas Press 1953.
Friede, R. L., van Houten, W. H.: Relations between post-mortem alterations and glycolytic metabolism in the brain. Exp. Neurol. **4**, 197—204 (1961).
Hager, H., Hirschberger, W., Scholz, W.: Electron microscopic changes in the brain tissue of syrian hamsters following acute hypoxia. Aerospace Med. **31**, 379—387 (1960).
Hills, C. P.: Ultrastructural changes in the capillary bed of the rat cerebral cortex in anoxic-ischemic brain lesions. Amer. J. Path. **44**, 531—543 (1964).
Hossmann, K.-A., Olsson, Y.: Suppression and recovery of neuronal function in transient cerebral ischemia. Brain Res. **22**, 313—325 (1970).
Kabat, H., Dennis, C., Baker, A. B.: Recovery of function following arrest of the brain circulation. Amer. J. Physiol. **132**, 737—747 (1941).
Karnovsky, M. J.: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. **27**, 137A (1965).
Kowada, M., Ames III, A., Majno, G., Wright, R. L.: Cerebral ischemia I. An improved experimental method for study; cardiovascular effects; and demonstration of an early vascular lesion in the rabbit. J. Neurosurg. **28**, 150—157 (1968).
Lindenberg, R.: Morphostatic necrobiosis. Investigations on the nerve cells of the brain. Amer. J. Path. **32**, 1147—1177 (1956).
— Patterns of CNS vulnerability in acute hypoxaemia, including anaesthesia accidents. In: J. P. Schadé and W. H. McMenemy (Eds.): Selective vulnerability of the brain in hypoxaemia, p. 189. Oxford: Blackwell Scientific Publ. 1963.
McEwen, L. M.: The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and Quabain. J. Physiol. (Lond.) **131**, 678—689 (1956).
Patton, H. D., Amassian, W. E.: Single and multiple-unit analysis of cortical stage of pyramidal tract activation. J. Neurophysiol. **17**, 344—363 (1954).
Venable, J. H., Coggeshall, R.: A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**, 407—410 (1965).
Webster, H. de F., Ames III, A.: Reversible and irreversible changes in the fine structure of nervous tissue during oxygen and glucose deprivation. J. Cell Biol. **26**, 885—910 (1965).

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