Ultrastructural Alterations in the Dog Brain After Profound Hypothermia Induced by Extracorporeal Circuit

BY JAMES H. HARRIS, PH.D., M.D.* AND JAMES T. ROBERTSON, M.D.

Abstract:
This study has demonstrated that hypothermia with or without circulatory arrest produced ultrastructural alterations in the dog brain. Fluid accumulation in the perivascular area and in the neuropil persists for at least seven days. Neurons in all areas of the brain studied are quite resistant to fluid alterations. Minimal changes occurring in neurons appear to be reversible from these studies with no neuronal necrosis, even with cooling to 10°C. Endothelial cells and oligodendrocytes exhibit essentially normal morphology in all experimental groups. Astrocytes and their processes undergo the most severe alterations, with cellular changes greater in dogs subjected to 60 minutes than to 30 minutes of cardiac arrest, and progressive degrees of damage occurring with increases in postpumping time prior to sacrifice. Our studies indicate cytological damage increases for longer periods posthypothermia than earlier reports which noted a resolution at 72 hours of the edema observed at 24 hours. We have demonstrated with the electron microscope (EM) that edema persists for at least seven days.

The application of new developments in the pumping technique such as improved filtering of microemboli may reduce the pathological alterations described in this study and aid the clinical management of patients subjected to hypothermia.

Additional Key Words
brain edema
cardiovascular bypass
cerebral circulation
cold effects
perivascular edema
electron microscopy

Introduction
Hypothermia with or without circulatory arrest has proved to be a valuable adjunct for cardiac and brain surgery. As a result of difficulties in the clinical application of the technique, a large number of research labs have explored the safe period of circulatory arrest and pathological changes resulting from hypothermia. Many of the early studies of hypothermia were done in smaller animals, such as rats and rabbits. However, most of the investigations in the last 15 years have used cats and dogs.

Alterations in the blood-brain-barrier by hypothermia as shown by fluorescein dye and other studies have implicated the astrocytes in the development of cerebral edema. Other investigators have shown alterations of neurons or glial cells or both, with damage persisting from 48 hours to one week. Perivascular edema was noted in dogs two weeks after hypothermia. The only electron microscopic (EM) study of brain hypothermia showed swelling of glial processes, choroid plexus epithelium and occasionally endothelium after ventricular perfusion. Polyvinyl pyrrolidine (PVP) granules (100 to 200 Å) were observed to traverse tissues from the ventricle to the capillaries.

The diversity of experimental techniques has led to some disagreement about the type of cell damage induced by hypothermia, how long the alterations persist, and whether circulatory arrest leads to more extensive damage than hypothermia alone. Because these conclusions (with one exception) were based on observations with the light microscope, it suggested to us that an electron microscopic study of the problem was indicated. There have been EM studies of cold-induced edema which have broadened and clarified concepts of cerebral edema following cold injury. However, these studies provided no pragmatic information regarding use of and damage induced by extracorporeal hypothermia such as that commonly employed by neurosurgeons. The present project was undertaken to delineate the sequence of ultrastructural alterations observed in several areas of the brain in an experimental design which closely simulates clinical reality.
Methods
Healthy male dogs weighing 20 to 22 kg were used in this study. The dogs were prepared for hypothermia induction by use of a pump-oxygenator and a heat exchanger. Esophageal and rectal thermometers monitored "core" body temperatures. Experience in this lab for several years has shown that brain temperatures usually vary only 1 to 4° above esophageal temperatures and not infrequently are lower than esophageal temperatures.

Dogs were divided into three groups as shown in table 1: control, 30 minutes circulatory arrest, and 60 minutes circulatory arrest. The circulation was arrested by shutting off the pump when the desired temperature was reached. The control group was further subdivided into three classes: normal dogs, dogs pumped cold and killed, and dogs pumped cold without circulatory arrest staying on the pump 60 minutes and then rewarmed. These were killed 24 hours later. At the specified time posthypothermia dogs were anesthetized with sodium thiopental and maintained on a respirator with methoxyfluorane in a mixture of 95% O₂ and 5% CO₂ until cardiac arrest.

A thoracotomy was performed and consistent fixation of the brain with glutaraldehyde perfusion was accomplished by using a Y-tube, allowing simultaneous perfusion of the brachiocephalic arterial trunk and the left subclavian artery. Hemostats or ligatures were placed on both subclavians distal to the origin of the vertebrals and on the internal thoracic arteries. Cross-clamping vessels proximal to each cannula prevented backflow of the perfusate. Venous drainage was allowed to enter the right auricle.

Rheomacrodex (500 to 1,000 cc) at 28 to 32°C was used to rapidly (three to four minutes) wash out the vasculature (until clear) prior to perfusion with 1,000 cc of cold (10 to 15°C) 6% glutaraldehyde buffered with Sorenson's phosphate (pH 7.4). The cold perfusate was utilized in an attempt to minimize the rapid autolysis of nerve cells subjected to hypoxic conditions. The glutaraldehyde was infused from a height of 50 to 60 cm with gravity being the only pressure utilized. Total time of glutaraldehyde perfusion usually was 35 to 45 minutes to insure thorough fixation of the entire brain. The perfused brains were removed and examined closely to confirm thorough fixation of the gross specimens.

The perfused brains were sliced frontally and 1-mm samples were taken from six areas: (1) cerebellar cortex — culmen or lobus centralis, (2) occipital cortex — gyrus splenialis or cinguli, (3) frontal cortex — gyrus cinguli, (4) pons — left or right nuclei pontis, (5) caudate — nucleus caudate corpus, (6) thalamus — nucleus lateralis or medialis anterior. Samples were placed in buffered glutaraldehyde for an additional 15 minutes, rinsed in buffer and postfixed in OsO₄ for two hours. Dehydration in a graded series of ethanol was followed by infiltration of the epoxy resin, DER 334.29,30 Thick sections (1.0 μ) were stained with paragon and examined. Thin sections with silver interference colors were cut with a Porter-Blum MT-2 ultramicrotome. Sections were picked up on 200 or 400 mesh grids and stained with uranyl acetate and lead citrate.29 Observations were made with a Phillips 200 electron microscope and micrographs were taken on Kodak EM film. An Omega D-2 enlarger with a point light source was used for printing on Kodak F-3 to F-5 papers.

Results
LIGHT MICROSCOPY
Light micrographs of representative areas in cerebellar cortex and thalamus (figs. 1 and 2) did not reveal alterations in structure like that observed by other investigators such as "infarct necrosis" and glial proliferation3 or glial and neuronal swelling.7,14

ELECTRON MICROSCOPY
Control animals exhibited the classical morphological pattern of brain tissues described by other investigators.28 The pump cold and sacrifice control animals revealed normal morphology of the frontal cortex (fig. 3) and caudate (fig. 4) and minimal dilata-

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Electron Microscopic Studies of Dog Hypothermia</th>
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<tbody>
<tr>
<td>No. dogs</td>
<td>Experiment</td>
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<td></td>
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<tr>
<td>5</td>
<td>Circulatory arrest</td>
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<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td>4</td>
<td>Circulatory arrest</td>
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<tr>
<td>6</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>1. Normal (2)</td>
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<td></td>
<td>2. Cool and sacrificed (2)</td>
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<td></td>
<td>3. Cool with no arrest (2)</td>
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**FIGURE 1**
Thick sections from control animals. a and b are cerebellar cortex and thalamus from normal dogs. c and d are sections from the same areas of a dog cooled without circulatory arrest.

**FIGURE 2**
Thick sections of experimental animals a and b are thalamus and cerebellar cortex from dogs arrested for 30 minutes and killed at 48 and 72 hours. c and d are cerebellar cortex and thalamus from dogs arrested for 60 minutes and killed at 48 and 72 hours. Note the lack of perivascular edema.

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tion of the endoplasmic reticulum in Purkinje cells (fig. 5). The pump cold control dogs without circulatory arrest were killed at 24 hours. Sections of frontal cortex, thalamus and pons (figs. 6 to 8) are generally normal, but the micrographs of thalamus show decreased electron opacity in perivascular processes, specifically astrocytes. There is no apparent increase in the intercellular spaces in areas adjacent to the swollen and disrupted astrocytes (fig. 7).

Experimental animals were subjected to 30 minutes' or 60 minutes' circulatory arrest and killed as shown in table 1. A summary of alterations observed in each region at each time interval is shown in table 2.

Within six hours after cooling there is a definite increase of fluid within the pericapillary region of the frontal and cerebellar cortices. This fluid is restricted mainly to the astrocytic footplates and processes (fig. 9). Within the thalamus, swollen astrocytic processes are noted adjacent to capillaries (fig. 10), but this is not apparent in sections of the pons (fig. 11).

Twenty-four hours after cooling, the ultrastructural alterations have become more pronounced and can be observed at some distance from the capillaries. There is perivascular fluid accumulation in the pons (fig. 12), disruption of astrocytic processes (fig. 13), and fluid uptake by adjacent cell processes (fig. 14) which may be dendrites (note synapse). The axons within the neuropil and oligodendrocytes appear to be resistant to fluid uptake.

After 48 hours all regions of the brain show some edema within the neuropil with the pons showing the least alteration. Neuronal organelles in the thalamus (fig. 15), frontal and occipital cortices show dilatation with poor preservation of mitochondrial cristae. The caudate shows fluid accumulation in the perivascular area and in the neuropil, with essentially no alteration of neuronal organelles even with perineuronal fluid accumulation (fig. 16). Multiple micrographs show greatest fluid accumulation in the perivascular area with diminution of fluid in distant areas.

By 72 hours, there is extensive fluid accumulation not only in the pericapillary region, but also...
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throughout the neuropil. The pons, which exhibited minimal alterations to this point, shows large perivascular fluid accumulations (fig. 17). The caudate shows similar alterations. Sections of frontal and cerebellar (fig. 18) cortices show distended cell processes (both glial and dendritic). Large spaces are formed by lysis of cell processes and coalescence of areas in the occipital cortex (fig. 19).

Perivascular edema is still marked seven days after hypothermia with moderate fluid accumulation in the neuropil (fig. 20). At higher magnification vesicles in myocytes exhibit the same granularity and electron opacity as basement membranes (fig. 21). Occipital and cerebellar cortices exhibit less severe alterations than that observed at earlier intervals. An unusual observation in the cerebellar cortex (fig. 22) reveals two swollen astrocytic processes on either side of an apparently “resistant” astrocyte with a relatively compact cytoplasm. Lamellar bodies (fig. 23) have been observed in dendrites and neuronal soma in multiple samples of different areas of the brain. They are not totally unlike the negative images of mitochondria, but have no circumscribed membranes and appear simultaneously with normal mitochondria in the same cell. A possible relationship to annulate lamellae is unclear.

Discussion

There is general agreement that hypothermia produces little brain damage if adequate tissue oxygenation is maintained by artificial respiration. It has been reported that in experimental animals profound hypothermia produces only diffuse reversible neuronal swelling, some cerebral edema in the white matter,

TABLE 2
Pathological Alterations in the Hypothermic Dog Brain*

<table>
<thead>
<tr>
<th>Sacrifice</th>
<th>Thalamus</th>
<th>Caudate</th>
<th>Pons</th>
<th>Cerebellar cortex</th>
<th>Occipital cortex</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>PVE</td>
<td>As</td>
<td>M</td>
<td>PVE</td>
<td>—</td>
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<td>24 hours</td>
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<td>PVE</td>
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<td>PVE</td>
<td>As</td>
<td>PVE</td>
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*Alterations observed less frequently included: changes in density of the basement membrane, presence of lamellar bodies, synaptic vesicle arrangement.

Abbreviations: PVE—perivascular edema, As—astrocyte swollen or lysed, D—dendrites, N—neurons, A—axons, M—mitochondria, V—vesicles.
FIGURE 3
Cerebellar cortex of pump cold and sacrifice animal. X 13,750.
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FIGURE 6
Frontal cortex neuron from hypothermia dog without arrest. X 22,750.
FIGURE 7

Thalamus of hypothermia dog with no arrest X 22,750.
and a reactive astrocytic proliferation. The Purkinje cells of the cerebellum seem to be the most vulnerable to hypothermic alterations. Cerebral edema and perhaps neuronal swelling apparently can be produced by hypothermia alone and by its injurious influence on the blood-brain-barrier. Kaplan and Quie attributed cerebral damage after circulatory arrest in deep hypothermia to capillary thromboses resulting from such factors as platelet aggregation intensified by increased blood viscosity and stasis.

The controls in our study were designed to allow us to separate the alterations produced by cooling itself and extended cooling without circulatory arrest. Since no changes were observed in pump cold and sacrifice controls, the possibility of pumping effects (dilution, denaturation of plasma proteins, and a slight hemolysis) and of cellular changes during cooling probably can be excluded. During the rewarming phase of hypothermia, cellular changes may occur as a result of the earlier cooling. In fact, dogs pumped cold with no circulatory arrest do exhibit structural alterations. The experimental groups enabled us to specify the changes resulting from hypothermia and circulatory arrest in animals when they are sacrificed six hours to seven days later. Although a number of photomicrographs were taken from thick sections of tissue, there was no pathology observed comparable to that reported by other investigators such as glial proliferation and neuronal necrosis or glial and neuronal swelling. One might speculate that since we employed fixatives for electron microscopy which are known to be superior to those used for routine histology, better fixation reduced the pathological alterations observed by others with the light microscope. The question also arises whether the changes observed in this study were pathological or artificial. However, the alterations were consistent in experimental animals and were a logical extension from histopathological studies.

The most severe pathological changes observed at the ultrastructural level in each group studied were observed in the cerebellar and occipital cortices, although the authors recognize the danger in extrapolating from electron micrographs to the gross or clinical level of observation. It is not understood whether this represents a true differential susceptibility of these regions to cooling (which is doubtful) or simply a deficiency in the perfusion fixation technique due to regional modifications in the microcirculation (which is more probable). One recent development which may obviate difficulties in hypothermia perfusion technique is Patterson’s improved filter for use in cardiopulmonary bypass techniques. Another interesting aspect of this study is the variation of the ultrastructure morphology in tissue sections cut from the same block of tissue and apparently related to proximity to circulation. Some areas show almost normal morphology. Another area only a few micra distant may show gross distortion of the neuropil in the perivascular area with partial to complete disruption of the astrocyte. This begins with the footplates adjacent to the capillary basement membrane and spreads in a centrifugal pattern. It is necessary to realize that alterations occur in three dimensions and the pathological details in a given area are a composite result of fluid and solute change in one to three or more closely adjacent capillaries. It is not clear whether the fluid observed is a transudate or an ultrafiltrate, although it is known that 100 to 200 A particles cross the capillary wall after hypothermia. Filaments of protein and/or lipid are present in the edematous space and may arise from the vascular lumen or from degradation of astrocytic cytoplasm into macromolecular fragments. Use of ferritin or horseradish peroxidase in our experiments would have enabled us to specify the size of particles transported.

FIGURE 8

Pons of hypothermia dog with no arrest. Note myelin figure at inner lamina of sheath. X 30,000.
Perivascular edema in frontal cortex after 24 hours. X 30,000.
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FIGURE 10
Thalamus six hours after hypothermia. Note glycogen and astrocytic filaments. X 30,000.

FIGURE 11
Pons six hours later showing neuronal soma and neuropil. X 22,750.
The protoplasmic astrocytes are the first cells which demonstrate ultrastructural alterations following hypothermia. This probably is a nonspecific response, since other investigators have shown the responsiveness of astrocytes to multiple types of physical and chemical trauma. The astrocyte appears to demonstrate a protective function for neurons and can rapidly adapt to various insults. With periods of circulatory arrest or with increased intervals posthypothermia, there is an increase of pathological alterations in these cells. It is very difficult to differentiate whether these two parameters have an additive or synergistic effect.

Within six hours after hypothermia moderate amounts of fluid have traversed the capillary wall and can be observed in the footplates of astrocytes. This fluid crosses two structures, the endothelium and the basement membrane, but it was not determined if the fluid crosses at the observation site or at some distant point. There is a functional defect in these structures as evidenced by the extravascular fluid accumulation and shown by others using ferritin tracers. We have observed no corresponding ultrastructural pathology in the endothelium, although increased pinocytotic activity has been suggested by others.

We have observed decreased electron opacity of the basement membrane in different areas, but this has been an inconsistent finding. As the fluid enters the footplates of the astrocytes, the resultant dispersion of cellular organelles and dilution of the intracellular material lead to a decreased electron opacity of the cytoplasm. It is interesting that mitochondria, known to be sensitive indicators of fluid alterations, retain their normal morphology for so long. Fluid accumula-
tion occurs even when pericytes or smooth muscle cells form part of the capillary or arteriolar wall. There are little or no alterations in pericytes and myocytes surrounding the vascular channels, which is remarkable when one considers the possible fluid transfer across these cells. We have observed 600 Å vesicles at the periphery of myocytes which are present normally in smooth muscle cells. Some are observed free in the cytoplasm but have the same granularity and electron opacity as the basement membrane. It is interesting that if they are being extruded from the cell, they should be morphologically similar to the basement membrane. They have been observed in sections of pons, caudate, thalamus, and occipital cortex, and appear only in myocytes and not in endothelial cells or pericytes. If these vesicles are pinocytotic in nature, it is interesting to speculate why they are not observed in all cells of the capillary or arteriolar wall.

In the early stages of perivascular edema, the intercellular space is restricted to the 150 to 200 Å space observed in normal nerve tissue with routine fixation. No intercellular edema was observed in these tissues, even though it has been shown to be the earliest form of brain edema and intracellular edema occurs later. With progression of the edema, there is disruption of the astrocytic organelles and loss of integrity of the astrocyte cell membrane. This results in a perivascular intercellular space filled with membranous and cytoplasmic fragments of the disrupted footplates. In addition, 50 to 100 Å filaments of protein and/or lipid (macromolecular degradation products) are irregularly distributed within the edematous fluid. Due to the resistance of the neuropil and the capillary wall to massive fluid uptake, the fluid accumulates in the
Swollen dendrites (*) and perivascular edema in occipital cortex at 24 hours. X 50,000.

Slightly edematous neuronal soma in thalamus after 48 hours. X 22,750.
Perineuronal edema near capillary in caudate after 48 hours. X 22,750.
perivascular area and creates a large pathological lesion which is extravascular and extracellular.

With longer periods of time, the extravasated fluid passes along the astrocytic processes in centrifugal pattern from capillaries. Degrees of fluid accumulation and cellular alteration analogous to that in the perivascular area appear throughout the neuropil. These changes become more pronounced in the 24, 48, and 72-hour dogs and persist for at least seven days but appear no more severe than at 72 hours. This would suggest one or more of the following possibilities: (1) variation in tissue samples as described; (2) maximal pathological changes reach a plateau around 48 to 72 hours; (3) maximal alterations are attained and then reversible changes occur. A large group of animals subjected to very carefully controlled experimental conditions would elucidate which of the three possibilities above is the actual sequence of pathological alterations.

We have seen the relative resistance of oligodendrocytes and neurons to the uptake of extravasated fluid following hypothermia. However, once the damage to astrocytes becomes pronounced, the neuronal dendrites and rarely axons show disruption of the normal cell organelles. The susceptibility of dendrites and resistance of axons to pathophysiological alterations after hypothermia is not apparent. In damaged processes mitochondria appear to be particularly vulnerable, but this may represent a nonspecific response to a hypoxic stimulus represented by the barrier of extravasated fluid in the perivascular area which may result from microembolization due to inadequate filtering during hypothermia. Neuronal soma remains remarkably resistant to fluid-induced
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FIGURE 18

Edematous processes in neuropil of cerebellar cortex at 72 hours. X 50,000.

changes, except for mild swelling of mitochondria or dilatation of golgi and endoplasmic reticulum cisternae. Neurotubules and filaments are not disrupted with progressive increase in intracellular fluid; however, these organelles appear more widely dispersed in the cytoplasm. The synaptic vesicles are quite resistant to alterations in size or shape, but their arrangement in the axon terminal can be changed. Most commonly one observes vesicles in a compact honeycomb (paradoxical) or widely dispersed (logical) pattern in the axon terminal subjected to fluid engorgement. Our studies show no obvious neuronal necrosis following profound hypothermia and the changes which do occur in neurons appear to be reversible. Further detailed studies should amplify and confirm our present observations and conclusions.

In general, the severity of cellular changes are greater in dogs subjected to 60 minutes than to 30 minutes of cardiac arrest, and progressive degrees of damage are observed with increases in postpumping time prior to sacrifice. Our studies indicate cytological damage increases for longer periods after hypothermia than earlier reports which noted a resolution at 72 hours of the edema observed at 24 hours. We have demonstrated with the electron microscope that edema persists for at least seven days.

The application of new developments in pumping technique such as improved filtering of microemboli may reduce the occurrence of the pathological alterations described in this study and aid the clinical management of patients subjected to hypothermia induced by an extracorporeal circuit. Prophylactic steroid therapy also has recently been shown to have therapeutic value in cold-induced edema.
FIGURE 19

Occipital cortex at 72 hours after 60 minutes' arrest. X 22,750.
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FIGURE 21
Higher magnification of 600 Å vesicles in myocytes. X 96,750.

FIGURE 20
Edema in perivascular area and in neuropil after seven days in thalamus. Note vesicles in myocytes. X 10,250.

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"Resistant" astrocyte in perivascular region of cerebellar cortex at seven days. X 22,750
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FIGURE 23
Lamellar bodies (see text) coexist with mitochondria in cerebellar cortex at seven days. X 22,750

Acknowledgments
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