Reversibility in blood-brain barrier, microcirculation, and histology in rat brain after decompression

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Nohara A, Yusa T. Reversibility in blood–brain barrier, microcirculation, and histology in rat brain after decompression. Undersea Hyperbaric Med 1997; 24(1)15–21.—To examine the changes in blood–brain barrier (BBB), cerebral microcirculation, and histology from 15 min to 72 h after decompression, 90 rats were exposed to experimental compression to 6 atm abs air for 90 min and subsequent rapid decompression. The disruption of BBB was examined by Evans blue extravasation. The cerebral microcirculation was demonstrated by perfusion with India ink. The area stained with Evans blue and the regions of defective filling with India ink, observed immediately after decompression decreased in size with time and were undetectable 3–24 h after decompression. The edematous brain tissue with enlarged perivascular space and darkly stained nerve cells also decreased to the uncompressed control level 1–24 h after decompression. These reversible dysbaric changes, however, reappeared 48–72 h after decompression. The different mechanisms, the physicochemical effects of microbubbles, and the maturation phenomenon after temporary brain ischemia induced by dysbaric microbubbles may be involved in the brain damage after decompression sickness.

compression-decompression, air bubble, blood–brain barrier, cerebral microcirculation, neuronal death, rat brain

Decompression sickness (DCS) affecting the central nervous system (CNS) (type II DCS) (1) is still a serious problem in diving and is regarded as predominantly a spinal cord disease with infrequent cerebral involvement. Clinically, cerebral symptoms such as mental cloudiness, confusion, and feeling of weakness are still commonly reported in neurologic DCS, but these are usually transient (2).

A recent study using $^{99}Tc^{m}$-labeled hexamethyl-propyleneamine oxime and single photon emission tomography, however, revealed that the cerebral perfusion deficits were present in divers after incidents of type II spinal DCS and suggests that the brain may be extensively involved in type II DCS (3). Morphologic changes in the CNS that may be related to diving have also been reported (4,5). The mechanisms involved, however, are in dispute and it is not known whether the changes in the brain after neurologic DCS are reversible or structurally permanent.

The purpose of this study was to examine the changes in the blood–brain barrier (BBB), cerebral microcirculation, and histology in the brain of rats up to 72 h after experimental acute dysbaric exposure.

MATERIALS AND METHODS

The experiment was approved by the Animal Care Committee of the University of the Ryukyus, Faculty of Medicine.

We used male Wistar rats weighing 250–300 g that were allowed to eat and drink ad libitum. The animals were divided randomly into an experimental and a control group and kept at ambient pressure (10 rats). The experimental group and the control group were further divided into an Evans blue (EB) group into which EB was injected to examine the disruption of BBB, and an India ink group which received India ink to demonstrate the cerebral microcirculation.

The experimental animals were compressed, one at a time, to 6 atm abs on air for 3 min in an animal hyperbaric chamber (Hanyuda Iron Works, Japan, model P-4000, vol. 160 liters). After exposure to pressure for 90 min, the rats were rapidly decompressed to the ambient pressure at a rate of 1.67 $kg \cdot cm^{-2} \cdot min^{-1}$. The rats that died within 15 min after decompression were excluded from the study. The surviving rats were randomly assigned to the following subgroups, five in each subgroup, according to observation (killing) times after decompression. Thereafter the experimental animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.17 mg $\cdot g^{-1}$) and killed by perfusion-fixation at 0.25, 0.5, 1, 3, 6, 12, 24, 48, and 72 h after decompression. Perfusion-fixation was performed through the left cardiac ventricle with heparinized saline (200 ml), followed by 10% formal saline (200 ml) at a pressure of 90 mmHg. Ten uncompressed control rats were anesthetized and fixed in the same way as the experimental animals.

Rats that died before the assigned observation time were

excluded from the study, and other rats were included up to a total of five in both the EB and the India ink group.

Changes in permeability of cerebral blood vessels were examined in the Evans blue group (including five control rats) by intravenous injection of 2% EB saline (3.5 ml · kg^{-1}) into a tail vein 15 min before perfusion-fixation at each observation time after rapid decompression. The cerebral microcirculation was demonstrated by transcardiac perfusion with 50 ml of India ink (particles 0.6–0.8 μm in diameter) after perfusion-fixation in the rats of the India ink group (including five control rats).

The brains were subjected to the gross examinations of EB extravasation and the filling of vasculature with India ink on dorsal surface and at one-third and two-thirds excised coronal sections.

For histologic examination, the brains were placed in fixative for 24 h to stabilize within the cranium; thereafter they were removed and stored in the same fixative until processed further. Small blocks of brain tissue were dehydrated and then embedded in Epoxy resin. Successive 2-μm-thick sections were stained with toluidine blue and safranine. Other 30-μm-thick celloidin-embedded sections were stained by thionin (Nissl's stain).

Statistical analyses for the incidence of EB extravasation and the defective filling of vasculature with India ink were performed using χ^2 analysis with Fisher exact test. A probability value less than 0.05 was considered statistically significant..

RESULTS

Clinical manifestations: Within 5–10 min after rapid decompression, half of the rats that were subjected to 6 atm abs air compression-decompression developed typical symptoms of mild-to-severe respiratory distress with panting and gasping, and this symptom gradually passed. The 19 animals that died within 15 min after decompression were not included in this study. At 15 min after decompression, intravascular gas bubbles were also observed in several experimental animals at the time of perfusion-fixation. Thereafter, no rat died before the time assigned to killing for observation of findings up to 24 h after decompression. However, three rats died before the observation (killing) time at 48 and 72 h after decompression, respectively.

Changes in BBB and microcirculation: The duration Of disrupted BBB against EB and the defective filling of vasculature with India ink was determined by varying the interval between decompression from 6 atm abs and administration of EB and India ink. We did not observe the area stained with EB and defective filling of vasculature with India ink during the period 3–24 h after decompression. The results in a total of 90 experimental and 10 control rats are summarized in Table 1.

Fifteen minutes after rapid decompression, the extent of the area stained with EB and the extent of the vasculature not filled with India ink were observed. The incidence of these findings was significantly higher than each control group. None of the control brains had areas stained with EB or regions of defective filling with India ink. In some of the experimental brains, extravasation of EB was seen in the cerebral cortex and cerebellum (Fig. 1*A*). Defective filling with India ink was seen to be most prominent in coronal sections of the cerebral cortex and the hippocampus (Figs. 1*A* and 2*B*).

These early findings were reversible in experimental animals. The BBB was impermeable to EB 1 h after decompression. The filling of vasculature with India ink also returned to the uncompressed control level 3 h after decompression. The incidence of these reversible findings at 1 h for BBB disruption and 3 h for the microcirculatory disturbances was statistically significant compared with 15 min after decompression, respectively (Table 1).

However, the area stained with EB and the regions of defective filling of vasculature with India ink reappeared 48 and 72 h after decompression, respectively (Table 1 and Figs. 1*D* and 2*D*). The incidences of these reappearances of both derangements compared with that of previous time point was $P = 0.11$ by χ^2 analysis and $P = 0.22$ with Fisher's exact test.

Histologic findings: Fifteen minutes after decompression, light microscopy of Epoxy resin-embedded sections demonstrated edematous brain tissue in superficial cortex with enlarged perivascular space and darkly stained, degenerative nerve cells (Fig. 3*B*).

In spite of perfusion done, some blood cells were found

Table 1: Reversibility of Evans Blue Extravasation and Defective Filling With India Ink (no. of rat)

Times After Decompression	Extravasation of Evans Blue		Defective Filling With India Ink	
	present	none	present	none
Control	0	5	0	5
15 min	4	1	4	1
30 min	2	3	4	1
1 h	0	5	4	1
3 h	0	5	0	5
6 h	0	5	0	5
12 h	0	5	0	5
24 h	0	5	0	5
48 h	2	3	0	5
72 h	2	3	2	3

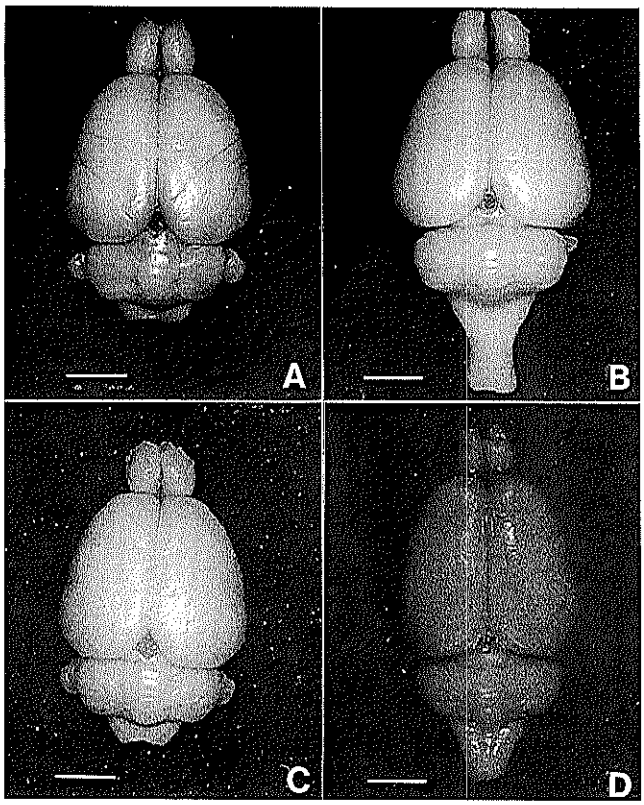

FIG. 1–Extravasation of Evans blue after 15 min (*A*), 1 h (*B*), 6 h (*C*), and 72 h (*D*) after decompression. Cortex and cerebellum were stained with Evans blue in *A* and *D*. Extravasation of Evans blue was not seen in *B* and *C*. *Original bar* = 5 mm

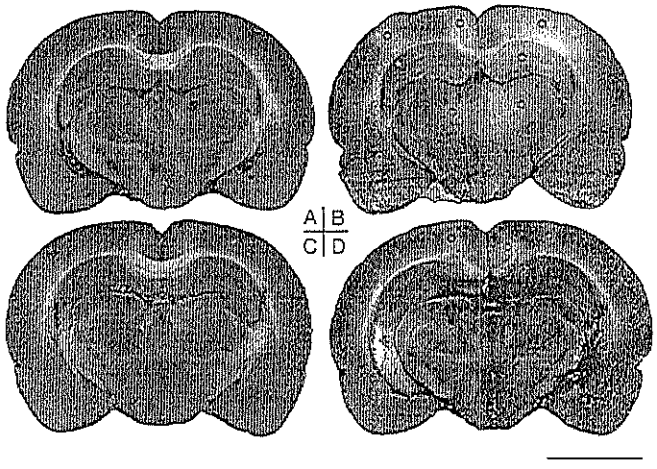

FIG. 2—Filling of India ink before (control, *A*) and 15 min (*B*), 48 h (*C*), and 72 h (*D*) after decompression. The defective filling of India ink (*circles*) was prominent in the cerebral cortex (*B* and *D*) and the hippocampus (*B*). No defective filling was found in *A* and *C*. *Original bar* = 5 mm.

in the lumen of the microvasculature, suggesting the defective perfusion through these areas (Fig. 4*B,D*). The edematous brain tissue with enlarged perivascular space was not seen 12–24 h after decompression.

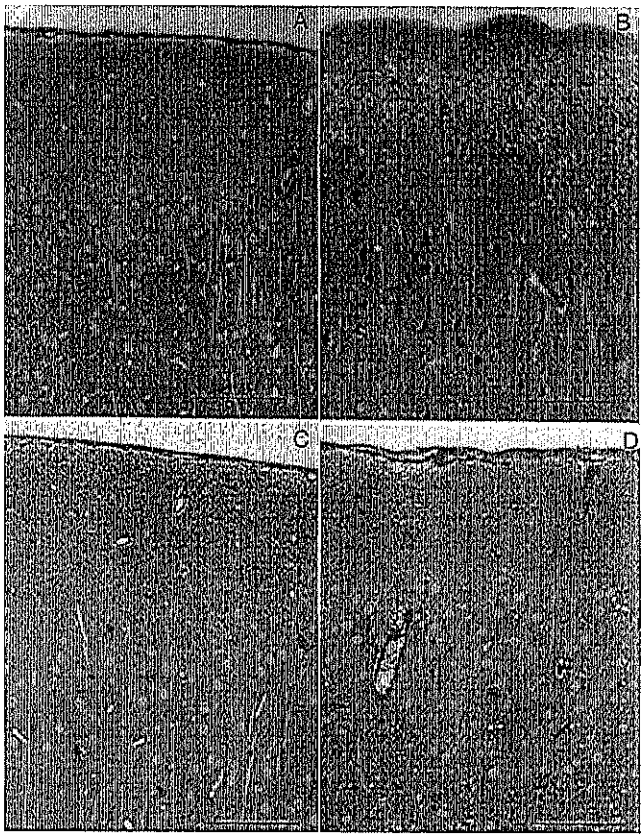

FIG. 3—Histologic findings in the cerebral cortex before (control, *A*) and 15 min (*B*), 24 h (*C*), and 48 h (*D*) after decompression. Edematous changes were seen in the surface of the cerebral cortex (*B*) and darkly stained nerve cells in the II–III layers (*B*) and perivascular space (*B* and *D*). *Original bar* = 100 μm.

Darkly stained neurons were observed frequently in the II–IV layers of the cerebral cortex, and especially pyramidal cells in these layers were darkly stained in rats killed from 15 min to 3 h after decompression (Figs. 5–7). These early derangements observed immediately after decompression decreased with elapsed time, and the number of dark neurons was decreased to the level of the control within 3 h after decompression. The perivascular space and a small number of darkly stained neurons reappeared in the cerebral cortex 48–72 h after decompression (Figs. 3, 4, 7).

DISCUSSION

In the brains of rats exposed to experimental compression to 6 atm abs air for 90 min and subsequent rapid decompression, the BBB, cerebral microcirculation, and histologic findings of brain tissue were immediately deranged. Although these finding reversibly returned to control levels within 1 to 3–6 h, these derangements reappeared between 48 and 72 h after decompression.

Dysbaric modification of BBB was reported by several investigators in a variety of animals (rat, rabbit, guinea pig) using various tracers such as trypan blue (6), Evans blue

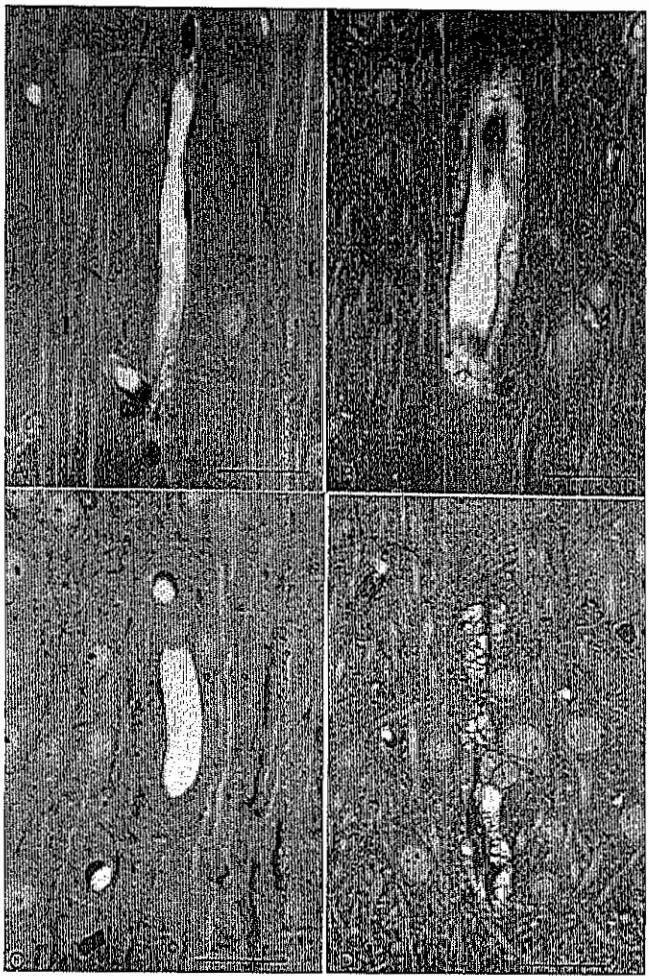

FIG. 4—Change of perivascular space before (control, *A*) and 3 h (*B*), 24 h (*C*), and 72 h (*D*) after decompression. The enlarged perivascular spaces were seen frequently in *B* and *D*. *Original bar* = 25 μm.

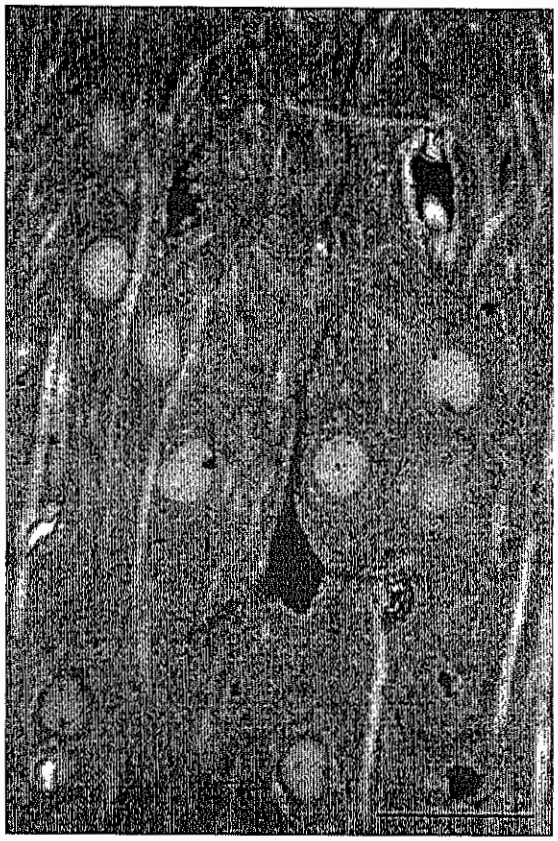

FIG. 5—Changes in nerve cells of cortex 3 h after decompression. The nerve cells darkly stained with toluidine blue and safranine. *Original bar: 25 μm.*

(7,8), horseradish peroxidase (as protein tracer) (9), and sodium fluorescein (as micromolecular barrier tracer) (10), and different experimental conditions. Few reports, however, described the detail of the reversibility of immediate BBB disruption after decompression. Gruenau et al. (7) studied the BBB effect of explosive decompression from a 30-min exposure to different hyperbaric N_2–O_2 mixtures in guinea pigs using Evans blue as a marker of BBB integrity. Brain staining occurred only when the hyperbaric mixture contained at least 2 atm abs N_2 and was reversed within 48 h after decompression, same as the rats exposed to the same condition in our experiment. In our study, most of the disruption of BBB became undetectable at 1 h after decompression.

In neurologic DCS, arterial air embolism, in situ gas formation in tissue, and venous occlusion by bubbles have been proposed as mechanisms of spinal cord damage, and arterial air embolism has been considered to be the main mechanism of brain damage (11).

The exact mechanism by which dysbaric exposure alters BBB permeability is obscure. Increased permeability of cerebral vasculature to dyes could be due to direct mechanical injury of blood vessels, ischemic changes, or permeability changes induced by chemical factors released or activated by microbubbles. Ischemia-hypoxia caused by embolization or circulatory disturbances, however, is not a likely pathogenic factor because anoxic states have been shown to produce no deleterious effects on cerebrovascular permeability within several hours of the early phases of ischemia (12).

In contrast, intravital inspection of pial arteries has shown that even a few seconds of intravascular gas exposure lead to almost immediate uptake of dye-albumin complex in the arterial vessel wall (13). The change in the permeability of cerebral vessels caused by microbubbles was also reversible. Hills et al. (14) injected microbubbles into the carotid artery of guinea pigs and observed the extravasation of albumin-binding trypan blue indicating BBB dysfunction until 2 h after injection, but undetectable after 3 h.

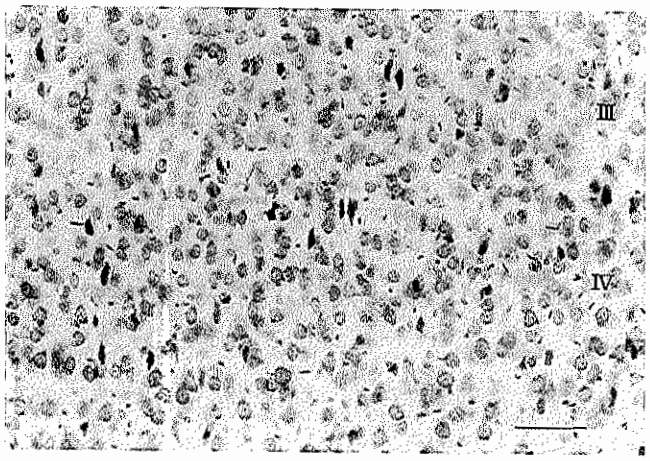

FIG. 6—Distribution of the darkly stained cells in layer III and IV of the cortex 3 h after decompression. *Original bar* = 50 μm.

Considering the onset time and reversibility of BBB disruption after decompression, temporal gaseous emboli that are retained for longer than 6 min (15) could increase the permeability of cerebral vessels, either by mechanical damage to the vascular endothelium (16) or by activating chemical agents such as serotonin or histamine (17) and by complement activation (18). The close relationship between intravascular bubbles and BBB disruption also has been suggested (6,7).

The regions of defective filling of cerebral vasculature with India ink (particles 0.6–0.8 μm in diameter) were demonstrated at almost the same area of EB extravasation immediately after decompression. These regions also decreased in size as the time elapsed, and were undetectable 3 h after decompression.

The primary event in gas embolism of the brain has been believed to be the obstruction of blood flow by bubbles trapped in arterioles, most likely in the intraparenchymal vessels at the junction of the gray and white matter (19). The intravascular bubbles could then lead to progressive processes of blood clotting by blood–bubble interaction, which worsens the mechanical blockage of the circulation (11).

Many bubbles that enter the brain circulation, however, pass rapidly through the arterioles and capillary beds and do not obstruct blood flow. The effect of different doses of intracarotid air on cerebral blood flow and brain function was investigated in rabbits by Helps et al. (20). After injection of the minimum dose of air necessary to cause embolism, but which passed through the vessels without any trapping, they observed that both cerebral blood flow measured by hydrogen clearance and neuronal function measured by cortical somatosensory evoked response slowly but progressively deteriorated over the next 90 min

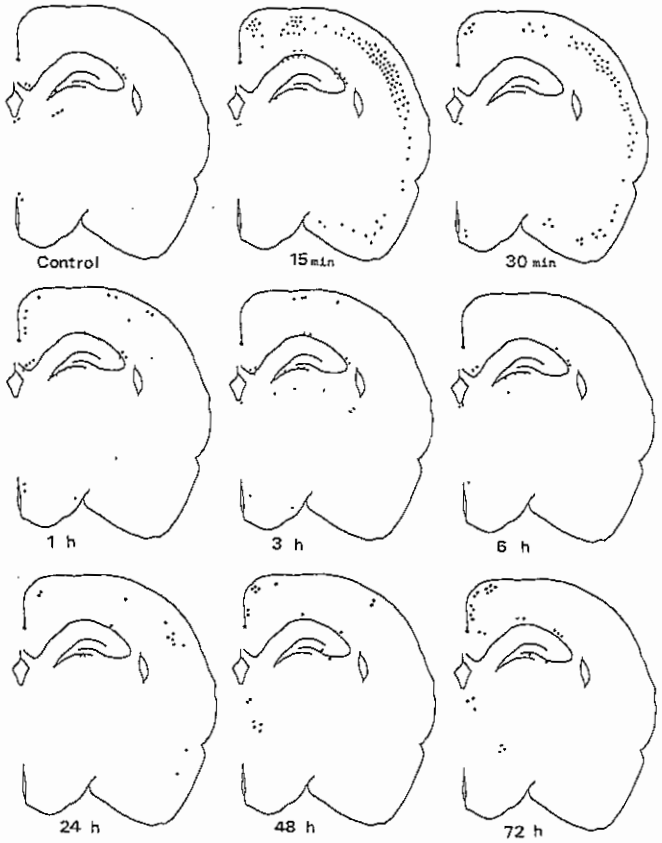

FIG. 7—Schematic drawing of the distribution of darkly stained nerve cells (dark neurons). *Each dot* represents a dark neuron found in three successive slices (30-μm-thick celloidin-embedded and stained by thionin, Nissl's stain).

and remained for over 3 h after injection. This persistent cerebral blood-flow reduction was consistent with the time course of disturbance of microcirculation in our study.

Since both the disturbance of microcirculation as revealed with India ink and EB extravasation immediately after decompression has similar reversibility in our experiment, the effects of edema induced by increased permeability or by autochthonous bubbles in tissue evolved in situ (21) most likely explain these reversible changes. This is supported by histologic findings of the edematous brain tissue with enlarged perivascular space immediately after decompression in our study. The perivascular space enlargement also decreased to control level within 6–12 h after decompression.

The transient appearance of darkly stained nerve cells after dysbaric exposure has not been reported previously. The number of these darkly stained nerve cells observed immediately after decompression also decreased with time. The number of dark neurons was markedly decreased after 3 h from decompression.

So-called dark neurons had previously been considered an indication of neuronal death or an artifact during processing the specimen (22). The dark neurons were

observed immediately after decompression, but reversibly decreased. Thus, this early appearance of dark neurons does not indicate neuronal death due to ischemic episode caused by microcirculatory disturbances. After production of autochthonous bubbles evolving in situ in the brain tissue by dysbaric exposure, the neuronal cells would have suffered transiently from some physical or biochemical changes that produce substances to make cells appear darkened by staining. The reversibility of neuronal hyperchromasia has also been reported in the rat cerebellar Purkinje cells after ischemia (23) and in the rat cortex after hypoglycemic brain damage (24).

The above-mentioned changes in BBB, cerebral microcirculation, and histology reappeared 72 h after decompression. The incidence of these reappearances of both derangements was not statistically significant. However, considering the usual circumstance where the incidence of the initial derangement in BBB and microcirculation would decrease progressively as time elapsed, the contingency of disrupted BBB and disturbed microcirculation at this time would be relatively high. The reappearance of these changes cannot be explained by the physicochemical effects of intravascularly formed microbubbles or a gas phase evolving in situ in the brain tissue, by which the early reversible changes could be explained.

It is well known that after a transient period (5–15 min)of ischemia followed by reperfusion, neurons in the CA1 region are degenerated over several days (25,26) and this cell death in the CA1 region has been termed "delayed neuronal death" by Kirino (25). The slowly progressive ischemic injury, however, was not limited to the hippocampus when ischemia was prolonged 30 min to 1 h. Cortical neurons, neurons in the dorso-lateral portion of the basal ganglia, and neurons in the diencephalon also showed slowly progressive ischemic injury 24 h after restoration of blood flow. These progressive injuries after recirculation were defined as the "maturation phenomenon" by Ito et al. (27). Beside the histologic change, the principle of maturation phenomenon was recognized in postischemic BBB and various biochemical parameters after reperfusion from less than 1 h of ischemia, depending on the intensity of the ischemia (28). Thus the maturation phenomenon observed in this study could result from transient ischemia due to microcirculatory disturbances caused by intravascularly formed microbubbles or autochthonous bubbles in situ in brain tissue.

The reversible changes in BBB, cerebral microcirculation, and histology in the brain of rats after acute decompression in our study could explain why the clinical finding that cerebral symptoms seen in divers immediately after decompression are usually transient. Moreover, the reappearance of derangements in BBB, cerebral microcirculation, and abnormal histology, including reappearance of dark neurons, could explain the reported finding of perfusion deficits caused by small vessel blockage using isotopic scanning (3).

We thank Dr. Yukio Hirata, Department of Anatomy, for his comments on histologic findings.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (no. 06671542).—*Manuscript received March 1996; accepted December 1996.*

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