

Full Paper

## Effects of Mannitol on Ischemia-Induced Degeneration in Rat Hippocampus

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**Abstract.** Although mannitol has been used as an osmotherapeutic drug on brain injury, the clinical efficiency of the drug are still controversial. In the present study, we examined the effects of mannitol on the edema in a hippocampal slice due to brief ischemia. To evaluate the effects, we employed an image analysis system that consists of an infrared-differential interference contrast (IR-DIC) microscope, an infrared CCD camera, and a computer with custom-made software. By this system, severity of the edema can be quantified as the coefficient of variation (CV) of digitalized slice images. The dose-dependent improvement on the deteriorated hippocampal slices could be obtained by administration of mannitol (10, 50, and 100 mM) after 10-min ischemia. However, field excitatory postsynaptic potentials (fEPSP) in CA1 stratum radiatum, which disappeared during 10-min ischemia, were never recovered by mannitol after more than 20-min treatment. fEPSP were blocked by the effective dose of mannitol for morphological recovery, but the effects found to be reversible. Although we failed to find positive rescuing effects of mannitol on the synaptic activities after ischemia, the protective effects of the drug on ischemic edema may rescue the secondary damages around the infarct area.

**Keywords:** mannitol, ischemia, brain edema, hippocampus, image analysis

### Introduction

Since the survival of neurons in the brain depends on energy production owing to oxygen and glucose supplied by cerebral blood flow, severe dysfunctions associated with changes in many kinds of cellular environments are caused by a brief arrest of blood flow for several minutes. Ischemic edema is one of the most frequent brain symptoms seen following blood flow arrest and has been thought to be caused by acidosis due to accumulation of lactate and the disturbance of ionic homeostasis resulting from energy depletion (1–3).

Mannitol has been used clinically as an osmotherapeutic drug on brain injury. Its protective effects have been confirmed on ischemic brain injury in animal models (4–10). The main mechanism of the neuroprotective effect of the drug is the increase of osmotic

pressure in cerebral spinal fluid (CSF). Although mannitol cannot cross the blood brain barrier (11), it has a potent anti-edema action by drawing water from intracellular spaces into the intravascular space (12, 13). As a result, the brain has been thought to be rescued by lowering intracellular pressure, and thus by lowering intracranial pressure. Mannitol has also been implicated as being protective with nonosmotic cerebral effects. One of the nonosmotic mannitol effects is the protection against free radical-mediated ischemic cell damage (14–16). Moreover, mannitol protects the brain by reducing blood viscosity (12, 17, 18).

On the other hand mannitol has been reported to worsen the infarct volume after transient focal ischemia in rat cortex, caudoputamen complex, and hemisphere in rats (19). It was also reported that mannitol did not have any effect on energy metabolism in forebrain ischemia (20). Treatment with mannitol after ischemia was shown not to favorably alter histopathological

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outcome in the forebrain model nor did it reduce infarct volume, and treatment with mannitol before ischemia did not produce reductions in neuronal injury (16). Osmotherapy by mannitol on brain injury is still controversial. Real time detailed observation and electrophysiological recording of brain cells under ischemia and under administration of mannitol will give clearer information on the therapeutic efficacy of mannitol.

We have already developed a novel method for quantification of brain cell swelling in rat hippocampal slices (21). This quantification method is based on the coefficient of variation (CV) calculation of the light intensity of slice images, captured by an infrared CCD camera and an infrared differential interference contrast (IR-DIC) microscope. Using IR-DIC, clear, well-shaped brain cell morphology in the slices can be observed. IR-DIC images, during experiments, are automatically processed to calculate CV. Quantification of brain cell swelling using CV is based on the differences in visibility between the normal cells and the swollen cells. The decrease in CV was anticipated when edema formed. We demonstrated in the previous study that there was a close relationship among apparent tissue deterioration, light transmittance changes, and changes in CV and that CV is a reliable quantification index for edema formation of brain tissues (21).

We applied this method for testing the effects of mannitol on the hippocampal slices. Morphological changes in neurons during experimental ischemia and during administration of mannitol were observed in the hippocampal CA1 pyramidal neurons, which have been known to be the most vulnerable to brain ischemia.

## Materials and Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Tokyo University of Pharmacy and Life Science and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### *Hippocampal slices preparation*

Male Wistar rats (3–5-week-old; Japan Laboratory Animals, Tokyo) were anesthetized with ether and decapitated. Immediately after decapitation, the brain was quickly removed and dipped for 5 min in cold (4°C) artificial cerebrospinal fluid (ACSF) oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Composition of the ACSF is as follows: 124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM glucose. Transverse hippocampal slices with 300-μm thickness were made using a vibratome slicer

(ZERO-1; Dosaka, Osaka) in the 4°C ACSF, transferred to a keeping chamber containing continuously oxygenated ACSF, and incubated for 1–2 h at room temperature (22–25°C) to allow recovery before experiments.

### *Medium perfusion*

The hippocampal slices were held at the center of the recording chamber and submerged in the oxygenated ACSF. Inflow of the ACSF into the chamber was driven by a micro tube pump (MP3; Tokyo Rikakiki, Tokyo) at a flow rate of 2 ml/min (Fig. 1B). The temperature of the ACSF in the recording chamber was kept at 32.5 ± 0.5°C by a medium heating system (TC-324B; Warner Instruments Co., Hamden, CT, USA). Ischemic ACSF was made by replacing glucose with 2-deoxy-D-glucose and deoxygenating the medium by bubbling with 95% N<sub>2</sub>/5% CO<sub>2</sub>: 124 mM NaCl, 2.5 mM KCl, 26 mM NaHCO<sub>3</sub>, 10 mM 2-deoxy-D-glucose, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>. To examine the effect of mannitol on ischemic damage, slices were superfused with ACSF containing different concentrations of mannitol (mannitol ACSF) after ischemia. Mannitol was dissolved in ACSF to concentrations of 10, 50, and 100 mM.

### *IR-DIC microscope configuration and image acquisition*

To observe the morphology of pyramidal neurons and background images located at a depth of 10 to 50 μm from the surface of the slices, we employed an IR-DIC system that consisted of a microscope (E600FN-IR-DIC; Nikon, Tokyo) equipped with a water immersion lens (NA: 0.8; CFI Fluor 40×; Nikon), a 100-W halogen light source, and an infrared filter (>720 nm). IR-DIC images were detected using an IR-CCD camera with 640 × 480 pixels (C3077-78; Hamamatsu Photonics, Hamamatsu), displayed on a black and white monitor through a camera controller (C2741-60, Hamamatsu Photonics) and stored on videotapes with a video recorder (SVO260; Sony, Tokyo) for analysis. The level of the average transmitted light intensity of the control slice images in the normal ACSF was set at approximately 60% of the saturation level of the system. Neither change in the illumination light intensity nor video enhancement was made during the experiments. The analog video signals of the full-frame IR-DIC images were then fed into a personal computer (Model GP8, CPU: Intel Pentium 4 (1 GHz), RAM: 256 Mbytes, OS: Windows 98SE) through a video capture board (Frame Grabber IQ-V50PCI, Hamamatsu Photonics) with digitization of 8-bit resolution at 10-s intervals. The captured images were displayed on a computer screen, sequentially stored as TIFF files and renewed at every capture.

### *Image analysis for quantification of edema*

Under the IR-DIC microscope, the difference in the visibility between the normal and the swollen neurons in brain slices appears as the difference in the sharpness of visual contrast. To quantify the changes in image, we employed CV that is calculated from the average of light intensity and its standard deviation (SD) in the region of interest (ROI) ( $350 \times 300$  pixels) placed at the area covering the pyramidal cell layer in the hippocampal CA1 region according to the following equation: ( $CV = SD / \text{average}$ ) using a custom-made software. We have confirmed the reliability of the value as an indicator for the brain edema. For a more detailed explanation, please see our previous study (21).

### *Extracellular field potential recording*

Extracellular field potential was recorded simultaneously with the IR-DIC image acquisition. A glass pipette recording electrode (resistance of 1–5 M $\Omega$ ) filled with ACSF was placed in the CA1 stratum radiatum to monitor dendritic field excitatory postsynaptic potentials (fEPSP). Schaffer collaterals were electrically stimulated once every 10 s using a bipolar tungsten electrode placed in the stratum radiatum with rectangular pulses with a duration and an intensity of 0.1 ms and 0.2–0.5 mA, respectively, that were generated by an electronic stimulator (SEN-7103; Nihon Kohden, Tokyo) and an isolator (ISO-Flex; A.M.P.I., Jerusalem, Israel). Evoked fEPSP were recorded using an amplifier (MEZ-7101, Nihon Kohden), digitized at 4 kHz, stored, and analyzed in a personal computer using a MacLab/4s system (ADInstruments-Japan, Tokyo).

### *Chemicals*

All chemicals used were purchased from Sigma (St. Louis, MO, USA).

## **Results**

### *Dose-dependent morphological recovery by mannitol after ischemic edema*

Figure 1A shows typical IR-DIC images in the course of ischemic ACSF perfusion and in the course of mannitol ACSF administration after ischemia. Before the exposure to ischemic ACSF, the images of neuronal cell bodies and dendrites can be observed clearly. When the slice was exposed to the ischemic ACSF, which was deprived of oxygen and glucose, swelling in the cell bodies and the apical proximal dendrites of the pyramidal neurons became visually evident approximately 5 min after the exposure. The deterioration of the images progressed during the insult, finally resulting

in neuron shapes almost unrecognizable in IR-DIC images.

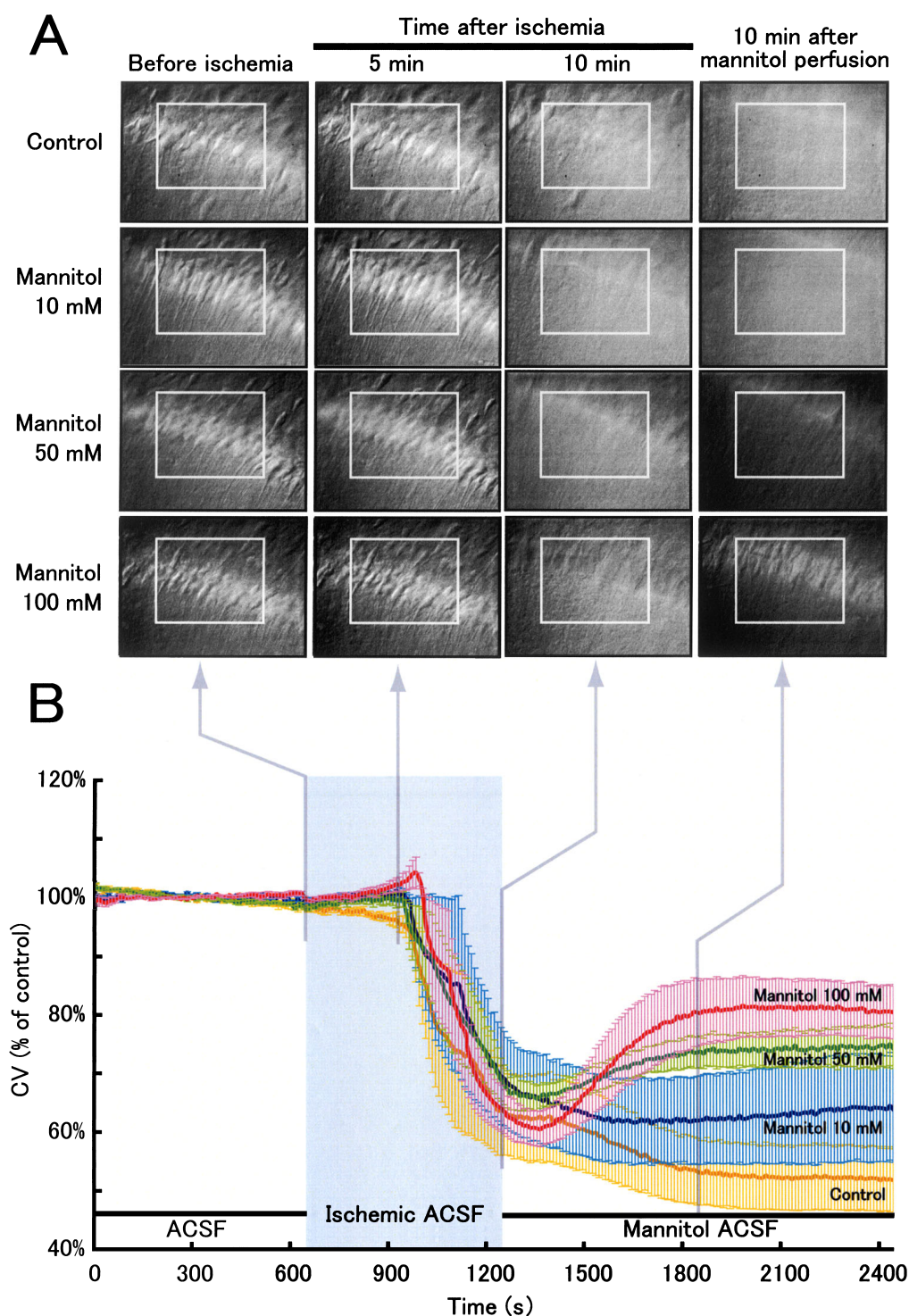
To quantify the magnitude of this morphological deterioration, we calculated CV by a real time image analysis system. The time courses of the morphological changes in different series of experiments are shown in Fig. 1B in which the CV values are normalized according to the average of control CV obtained before exposure to ischemic ACSF. During 0 to 5 min after ischemia, there was no apparent morphological change in all series (Fig. 1A), and there was no significant changes in CV value. After 5 min of ischemia, the CV in all series of experiments suddenly started to decrease. The obvious swelling of hippocampal cells can be seen in IR-DIC images after 10 min of ischemia (Fig. 1A) and CV decreased to  $67 \pm 2.7\%$  (S.E.M.) ( $n = 14$ ) (Fig. 1B).

After exposure to ischemic ACSF for 10 min, the medium was changed to normal ACSF or that containing various concentrations of mannitol (10, 50, and 100 mM). The morphological features of slice preparations and their CV values never recovered even after 20-min perfusion with normal ACSF. However, after 10 min of perfusion with ACSF with mannitol, partial morphological recovery was observed in a dose-dependent manner as shown in Fig. 1A (the last panels in each series of experiments) and Fig. 1B. Figure 2 shows the ratio of CV recovery after 10 min of mannitol ACSF perfusion. There were statistically significant CV recoveries in the preparations treated with ACSF containing 50 and 100 mM of mannitol. The effective concentrations of mannitol are consistent with the clinical doses of mannitol.

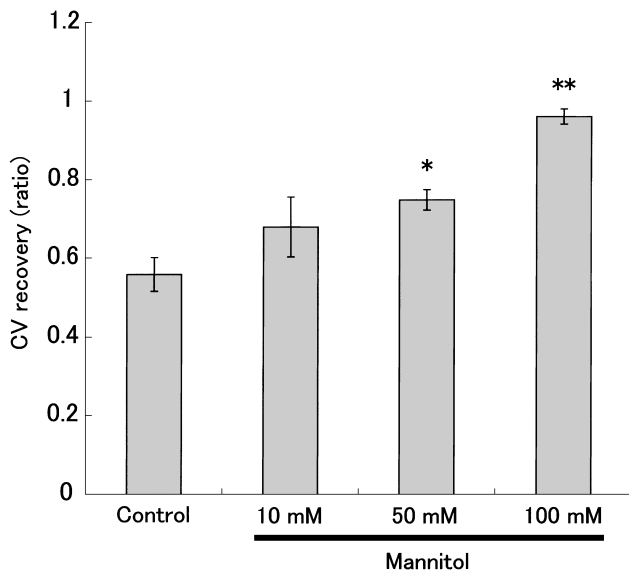
### *Electrophysiological evaluation of mannitol effect*

We made extracellular electrophysiological recordings in parallel with the CV analysis to examine whether morphological recovery is accompanied by any functional recovery. In those experiments, ACSF with 100 mM mannitol was administered after 10-min ischemic challenge (Fig. 3). Figure 3A shows the morphological changes of hippocampal cells and the changes of fEPSP recorded from CA1 stratum radiatum. Figure 3B shows the alteration of CV and slope of fEPSP.

In normal condition, cell bodies and dendrites of CA1 pyramidal neurons could be observed. CV in the normal condition was  $0.060 \pm 0.0008$  ( $n = 4$ ) (Fig. 3: A and B, a). fEPSP started to decrease gradually soon after the ischemic insult, suddenly turned to an increase at 230 s after the start of the insult (Fig. 3: A and B, b), and abruptly disappeared at 480 s (Fig. 3: A and B, c). It is notable that the timing of this disappearance of fEPSP coincided with the onset of the steep decreases in CV,



**Fig. 1.** The effects of mannitol on ischemic edema in the CA1 region of hippocampal slices. A: Typical IR-DIC images in the course of ischemic ACSF perfusion and in the course of mannitol ACSF administration after ischemia. Images from top to bottom indicate the concentration of mannitol perfused after ischemic insult: 0 (control), 10, 50, 100 mM. The arrows indicate the time-points during the experiment corresponding to those shown in panel B: From left to right: before, 5 and 10 min after the start of ischemic ACSF perfusion, and 10 min after mannitol ACSF perfusion. The white square in each IR-DIC image indicates the ROI in which optical parameters were quantified. B: Time course of changes in CV induced by ischemic insult for 10 min (shaded period) and reperfusion of mannitol ACSF are indicated in ratio with the mean  $\pm$  S.E.M. Each line indicates the concentration of mannitol in ACSF perfused after the ischemic trial: red line, 100 mM; green line, 50 mM; blue line, 10 mM; and orange line, control. Number of experiments are as follows: 100 mM,  $n = 4$ ; 50 mM,  $n = 4$ ; 10 mM,  $n = 3$ ; and control,  $n = 3$ .



**Fig. 2.** Dose-dependent effects of mannitol on ischemic edema. Each bar indicates the mean  $\pm$  S.E.M. of CV recovery ratio. CV recovery ratios were calculated from dividing CV 10 min after the mannitol perfusion by CV onset. Each bar shown is representative of four experiments. \*\* $P < 0.01$ , \* $P < 0.05$ , compared with the control (Fisher PLSD).

suggesting that loss of synaptic transmission is closely related to the initiation of marked morphological changes in neurons. At the end of 10-min ischemia, the IR-DIC image showed swelling of hippocampal pyramidal cells, and CV decreased to  $0.039 \pm 0.0010$  ( $n = 4$ ) (Fig. 3: A and B, d).

Perfusion by ACSF with 100 mM mannitol for over 900 s recovered the shapes of neuronal cell bodies and dendrites. CV value was significantly improved to  $0.049 \pm 0.0009$  ( $n = 4$ ;  $P < 0.001$ ,  $t$ -test). On the other hand, fEPSP never recovered (Fig. 3: A and B, e). After reperfusion of normal ACSF for 10 min, hippocampal cells swelled again and CV decreased to  $0.034 \pm 0.0011$ . Only the presynaptic fiber volley reappeared as shown in Fig. 3A, f (indicated by an arrow).

#### *Effects of mannitol for normal hippocampal synaptic transmission activity*

We tested the effects of mannitol on electrophysiological activities to confirm the effects of the drug on the normal synaptic transmission (Fig. 4 lower plot). The ACSF containing 100 mM mannitol depressed the slopes of fEPSP to  $21 \pm 4\%$  ( $n = 5$ ). After the normal ACSF reperfusion for 1500 s (indicated by an arrow), fEPSP recovered to  $101 \pm 30\%$ . In some preparations, fEPSP were found to be facilitated after reperfusion with normal ACSF. During the exposure to 100 mM mannitol ACSF, there was no significant morphological

change (indicated by CV) under IR-DIC images (Fig. 4, upper graph).

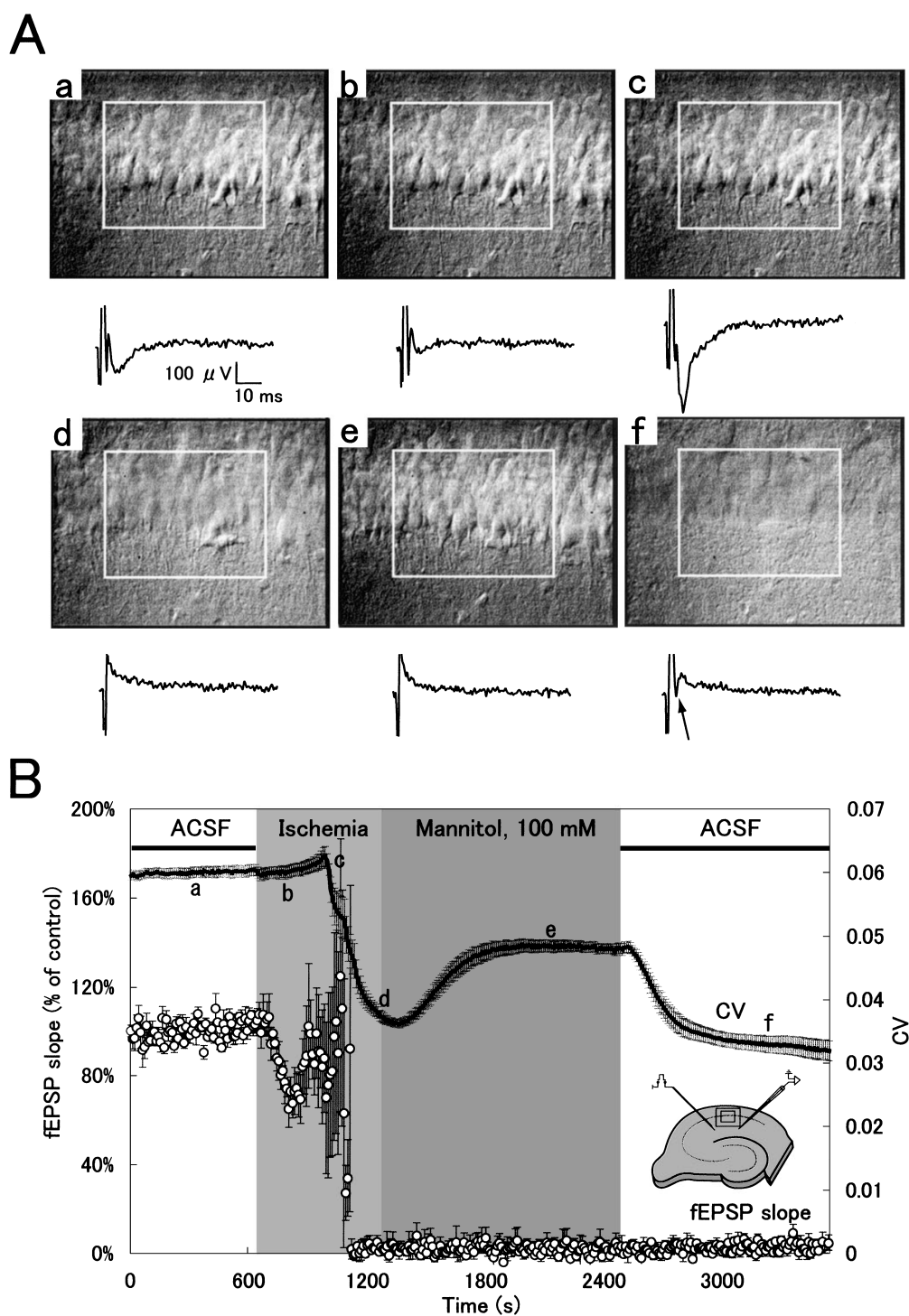
#### **Discussion**

Although mannitol has been used as an osmotherapeutic drug for brain injury, its therapeutic efficacy remains inconsistent. Some experimental data on focal ischemia demonstrate the adverse effects of mannitol on the brain (19). Most of reports on the effects of mannitol have been based on in vivo experiments, in which the effects of mannitol are evaluated by histological examinations based on fixed and stained brain preparations. In the present study, we examined the protective effects of mannitol on ischemic edema by a new method that enables us to detect the morphological conditions of cells in hippocampal slice preparations quantitatively. In some experiments, we simultaneously recorded extracellular fEPSP to see synaptic activity during and after the ischemic insult.

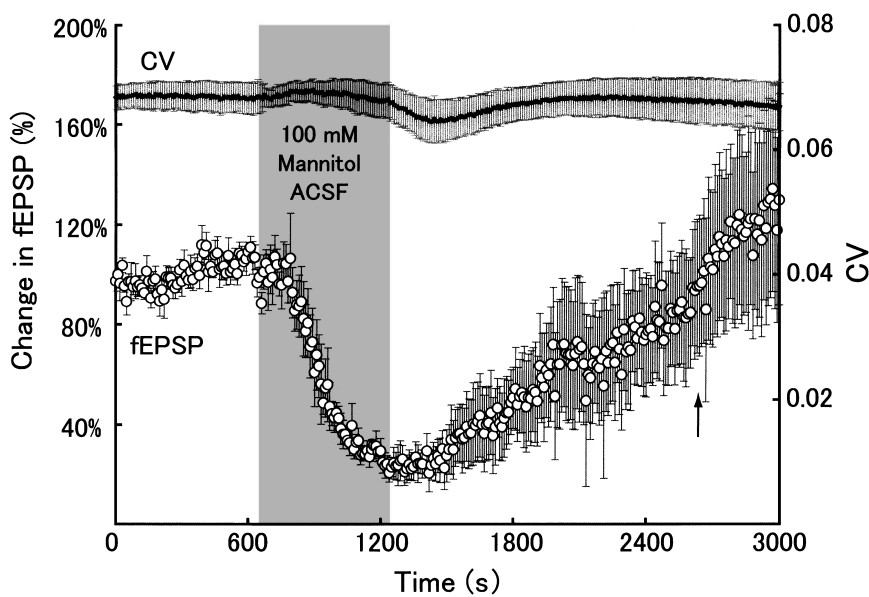
In our method, differences in the morphological features between the normal and the deteriorated neurons in brain slices before and after the ischemia are evaluated by statistical quantification of the IR-DIC images (21). Individual neurons under the normal condition are recognizable under an IR-DIC microscope because of the sharp visual contrast with the edge of cells being profoundly dark, while in contrast, the cell surfaces being bright. This property produces an uneven image of the focal area containing neurons. To the contrary, swollen neurons exhibit lower visual contrast, with the edge of the neurons being less dark and their surface being less bright, resulting in an apparently even image of the focal area. By calculating the CV value according to the procedure shown in Materials and Methods, we can investigate the time course of changes in cell morphology.

We can detect the drastic changes in cell morphology as a sudden decrease in CV value during the exposure to the ischemic condition (perfusion with ACSF deprived of oxygen and glucose). The effects of ischemia on cell morphology were recovered by post-administration of mannitol in a dose-dependent manner. The recovery obtained by 100 mM of mannitol seemed to be satisfactory from their morphological images and CV value.

To examine the protective effects of mannitol on hippocampal synaptic functions, we examined fEPSP. Previous investigations including ours have demonstrated that the membrane potential of CA1 pyramidal neurons during exposure to ischemic conditions showed a stereotyped response characterized by an initial hyperpolarization, followed by a rapid and sustained depolarization, called anoxic depolarization (22–25).



**Fig. 3.** The effect of mannitol on ischemic edema, quantitative optical parameters, and fEPSP in the CA1 region of hippocampal slices. **A:** IR-DIC images of the CA1 region (top) and field potentials (bottom). Marks a to f indicate the time-points during the experiment and correspond to those shown in panel B: a: control condition; b–d: 230, 480, and 1240 s after the start of ischemic perfusion, respectively; e: 920 s after the end of the ischemic insult; f: 600 s after 100 mM mannitol ACSF perfusion. An arrowhead in panel f indicates presynaptic fiber volley. The white square in each IR-DIC image indicates the ROI in which optical parameters were quantified. **B:** Time courses of changes in CV and fEPSP slope (open circle) are shown as the mean  $\pm$  S.E.M. Brighter shaded period: ischemic insult for 10 min. Darker shaded period: perfusion of 100 mM mannitol ACSF after ischemia. Period without shading: perfusion of normal ACSF. The average of fEPSP slope obtained at the first 10 min-control period of the recordings is represented as 100%. The data shown is the average of four experiments. The place where we put the electrodes, the area we observed (outer square), and the ROI (inner square) are indicated in the inset figure.



**Fig. 4.** The effects of mannitol on fEPSP and CV in the CA1 region of hippocampal slices. Time courses of fEPSP slope (open circle) and CV (solid line) during perfusion of 100 mM mannitol ACSF (shaded period) and wash-out with normal ACSF indicated as the mean  $\pm$  S.E.M. (CV:  $n = 3$ , fEPSP:  $n = 5$ ). The average of fEPSP for the first 10-min control period of the recordings is represented as 100%. An arrowhead indicates the time point at which the average of fEPSP slope recovered to 100%.

There are general agreements that in the initial hyperpolarization, an increase in  $K^+$  conductance is generated (26–28). The rapid depolarization has been shown to be dependent on  $Na^+$ ,  $Ca^{2+}$ , and  $Cl^-$  influx into the neurons (25). Although the morphological feature of the slice preparation recovered during the exposure to mannitol, fEPSP never recovered within 20 min of treatment by mannitol. Since we found that mannitol itself had strong inhibitory effects on the neurotransmission in the hippocampus, it might be difficult to examine the rescuing effects on the synaptic functions during the administration of mannitol. When mannitol was withdrawn from the medium after the 20-min mannitol administration, cell swelling, and decrease in CV value occurred within a few minutes of the withdrawal. Only the presynaptic fiber volley recovered after ACSF reperfusion, but fEPSP never recovered. Since the inhibitory effects of mannitol on fEPSP were reversible and rather facilitating effects were observed after washing out, the mannitol administration must be much longer to have rescuing effects on fEPSP.

In the present study, we failed to find any permanent recovery of deteriorated cells by mannitol as far as our experimental conditions are concerned. It is difficult to conclude the efficacy of mannitol treatment. Many reports have shown that mannitol reduces infarct volume in vivo (4–10, 29). However, in our report, no permanent recovery was found. One of the most important reasons of this contradiction is the difference between in vivo and in vitro experiments. We should note that the pharmacological effect of mannitol is

distinct in vivo and in vitro. In vivo, the intravascular mannitol absorbs interstitial edema fluid into the vascular lumen by its hyperosmolarity. In contrast, in vitro, as the slice contains no circulating blood, the interstitial mannitol only absorbs intracytoplasmic fluid content. In the acute stage of brain edema, swelling of astrocytes and neurons are much more remarkable than the increase of interstitial fluid in the extracellular space. There still remains the possibility that longer treatment with mannitol may cause neuronal recovery. We could show clear dose-dependent morphological recovery with mannitol for the first time in such a high temporal/spatial resolution. These results will clarify much more detailed mechanisms of the anti-edema effects of mannitol.

Mannitol may rescue the surrounding neurons around the damaged infarct area, called the penumbra area. Neurons in the penumbra area will secondarily be harmed by the increased intracranial pressure and by the leakage of intracellular fluid from broken cells. These neurons in the penumbra area can be rescued with the effect of reducing the volume of the first infarct area by mannitol. Further investigations on the protective effects of mannitol on ischemic cell damages are now in progress according to a protocol for examining the secondary effects of the focal ischemia.

Our new system reported here is easy to handle and has high reproducibility, and it can be quantitatively applied to patho-physiological studies on brain functions as well as pharmacological studies on quantitative evaluation of drugs for the treatment of brain injuries.



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