Original Article

Mannitol infusion immediately after reperfusion suppresses the development of focal cortical infarction after temporary cerebral ischemia in gerbils

Umeo Ito,[1,3] Yoji Hakamata,[2] Kazuhiko Watabe[1] and Kiyomitsu Oyanagi[3]

[1]Laboratory for Neurodegenerative Pathology, Department of Sensory and Motor Systems, Tokyo Metropolitan Institute of Medical Science, [2]Department of Basic Science, School of Veterinary Nursing and Technology, Faculty of Veterinary Science, Nippon Veterinary and Life Science University, Tokyo, and [3]Division of Neuropathology, Department of Brain Disease Research, Shinshu University School of Medicine, Matsumoto, Japan

Previously we found that, after temporary cerebral ischemia, microvasculogenic secondary focal cerebral cortical ischemia occurred, caused by microvascular obstruction due to compression by swollen astrocytic end-feet, resulting in focal infarction. Herein, we examined whether mannitol infusion immediately after restoration of blood flow could protect the cerebral cortex against the development of such an infarction. If so, the infusion of mannitol might improve the results of vascular reperfusion therapy. We selected stroke-positive animals during the first 10 min after left carotid occlusion performed twice with a 5-h interval, and allocated them into four groups: sham-operated control, no-treatment, mannitol-infusion, and saline-infusion groups. Light- and electron-microscopic studies were performed on cerebral cortices of coronal sections prepared at the chiasmatic level, where the focal infarction develops abruptly in the area where disseminated selective neuronal necrosis is maturing. Measurements were performed to determine the following: (A) infarct size in HE-stained specimens from all groups at 72 and 120 h after return of blood flow; (B) number of carbon-black-suspension-perfused microvessels in the control and at 0.5, 3, 5, 8, 12 and 24 h in the no-treatment and mannitol-infusion groups; (C) area of astrocytic end-feet; and (D) number of mitochondria in the astrocytic end-feet in electron microscopic pictures taken at 5 h. The average decimal fraction area ratio of infarct size in the mannitol group was significantly reduced at 72 and 120 h, associated with an increased decimal fraction number ratio of carbon-black-suspension-perfused microvessels at 3, 5 and 8 h, and a marked reduction in the size of the end-feet at 5 h. Mannitol infusion performed immediately after restitution of blood flow following temporary cerebral ischemia remarkably reduced the size of the cerebral cortical focal infarction by decreasing the swelling of the end-feet, thus preventing the microvascular compression and stasis and thereby microvasculogenic secondary focal cerebral ischemia.

Key words: astrocytic end-feet, mannitol infusion immediately after reperfusion, microvasculogenic secondary focal cerebral ischemia, suppression of focal infarction, temporary cerebral ischemia.

INTRODUCTION

After reperfusion following temporary ischemia, experimental animals undergo various post-ischemic neuronal changes, ranging from degeneration to recovery or death, and a focal infarction occurs once the threshold of the ischemic insult has been surpassed. The focal infarction had been thought to develop in the maturing neuronal necrosis due to progressive metabolic disturbances. Infarction is defined as a pan-necrosis of both glial and neuronal elements and is different from disseminated selective neuronal necrosis (DSNN) in that astrocytes also die during the development of an infarction.[1,2] Impairment of energy metabolism, as evidenced by reduced glucose utilization, progressive acidosis and ATP depletion,[1–3] has been

considered a pivotal player in the formation of an infarction. However, little is known about the mechanisms underlying the abrupt onset of astrocytic death and pannecrosis in the DSNN,[1–3] and a question remained unanswered: is the transition from maturing DSNN to the abrupt onset of a focal infarction in it continuous or not?

In our earlier studies, we found that the window for the threshold from DSNN to focal infarction after a temporary ischemic insult is very narrow and that a small increase in the ischemic insult induces an infarction once a critical threshold of the intensity has been met.[4,5] To observe the pathological changes just prior to the development of a focal infarction after restitution of the blood flow, we devised a modified new model in which a threshold amount of ischemia produces a focal cortical infarction in the left cerebral hemisphere.[4–6] Stroke-positive animals were selected by observing animal behavior during the first 10 min of left carotid occlusion performed twice at a 5-h interval. This method provided the threshold amount of ischemic insult needed to induce a unilateral cerebral cortical focal infarction in the region of maturing DSNN. Also this procedure drastically reduced the epilepsy-induced mortality rate of the animals.

In our last study,[7] using this model, we examined the mechanisms underlying the abrupt onset of focal infarctions in the maturing DSNN after temporary ischemia and found that transient but marked swelling of the astrocytic end-feet occurred 3–8 h after the return of cerebral blood flow. This swelling compressed the microvessels and induced stasis and microvasculogenic secondary focal cerebral ischemia, thus leading to the development of a delayed focal cortical infarction, which appeared at 12 h and reached a maximum at 72 h after the temporary ischemia, while microvascular compression by the swollen astrocytic end-feet disappeared in the infarction due to astrocytic necrosis, and then microvascular stasis disappeared.

Almost all of a dozen studies examined the effect of mannitol combined with other agents (cocktail therapy) applied before and during ischemia, for the purpose of protecting against an infarction induced by temporary clipping of a feeding artery to secure premature rupture during aneurysm surgery.[8–11] Also, Little[12] reported that mannitol infusion reduced vascular obstruction, as studied by carbon-black infusion, when given at the time of vascular occlusion in a permanent ischemia model. By ranging over numbers of literatures, no study has been found reported that mannitol infusion immediately after reperfusion following temporary ischemia reduced the infarct size.[8–11]

Here, using the same model as used in our last study, we examined the therapeutic effect of post-ischemic mannitol infusion performed immediately after the restitution of the cerebral blood flow to prevent the swelling of astrocytic end-feet, which we predicted would protect against microvascular compression and microvasculogenic secondary focal cerebral ischemia, thereby reducing the size of the focal infarction. In contrast to the clinical use of mannitol for treating ischemic brain edema, which reaches a maximum several days post-ischemia, in this study, we administered mannitol immediately after the return of the blood flow and long before the appearance of brain edema.

MATERIALS AND METHODS

Mongolian gerbils (*Meriones unguiculatus*, MGS/Sea. Kyudo Co. Ltd, Tosu, Japan) were handled in accordance with the ARRIVE guideline and the guideline of Animal Care and Use Committee of Nippon Veterinary and Life Science University in Tokyo, where the animal experiments were performed. The animals were numbered and grouped by an outsider using a table of random numbers.

The experiments were conducted measuring rectal temperature under controlled environmental conditions of 22°C to 23°C and 50–55% humidity. Under anesthesia with 3% isoflurane, we exposed the left carotid artery of adult male Mongolian gerbils (60–80 g: 11 weeks old) via a midline cervical incision. The left carotid artery was occluded for 10 min with a Heifetz aneurysm clip, and this occlusion was performed twice, with a 5-h interval between the two occlusions.[13]

Anesthesia was discontinued immediately after each cervical surgery and the behavior of conscious animals caged in the same room was observed. We scored the animals by observing their behavior during the first 10-min period of ischemia. Stroke-positive animals of about 30% were selected on the basis of a stroke-index score of greater than 10 points. During all procedures, animals were kept under the same conditions and all data were statistically significant even though there were small numbers of 3–5 animals in each group.[13,14]

We allocated the animals into the following four experimental groups: (A) sham operation (control group); (B) no treatment after ischemia (no-treatment group); (C) mannitol perfusion after ischemia (mannitol group); and (D) saline perfusion after ischemia (saline group). For the perfusion groups, 20% mannitol (1.2 g/kg body weight (BW); e.g. 0.36 mL for a gerbil weighing 60 g) or saline (0.36 mL for a gerbil weighing 60 g)[12] was applied twice via a cervical vein by using an infusion pump (KDS100, KD Scientific, Holliston, MA, USA). Each infusion occurred over a 15-min period, one that began immediately and the other, 4 h after the final 10 min of ischemia. The same anesthesia as above was administered during the perfusions in the same manner as described above. We then performed the measurements described below.

Measurement of infarct size

For the light-microscopic (LMS) studies, five animals per group were euthanized 72 or 120 h after the last ischemic insult via a 30-min trans-cardiac perfusion with 10% phosphate-buffered formaldehyde fixative. From each animal, three coronal paraffin sections of 3-μm thickness were prepared at 400-μm intervals after slicing in the coronal plane at the chiasmatic level (Face A). The sections were separately stained with HE or PAS, and the HE-stained sections were scanned onto a white paper and enlarged to 250×160 mm with 800 dpi resolution. The marginal lines of the infarct area were drawn by microscopically observing the HE-stained sections under 50 and $100 \times$ magnification. Similarly, we drew the marginal line of the cerebral cortex on each section. Using a digital planimeter (digitizer Measure-5, System Supply, Nagano, Japan), we measured the areas (in mm^2) of the infarcts and the cerebral cortices that had been scanned onto the white paper and subsequently calculated the area ratio of the infarct in each cerebral cortex as a decimal fraction. The average infarct area ratio of each group was then calculated.

Number of patent carbon-black-suspension-perfused (CBSP) microvessels

We examined the control, the no-treatment group at 0.5, 3, 12, and 24 h after the temporary ischemia (3 animals each), the mannitol group at 3 h (3 animals) and the no-treatment and mannitol groups at 5 and 8 h (five animals each). Under the same anesthesia, the gerbils were perfused for 30 s with 1.0 mL of carbon-black-suspension (Platina, Tokyo, Japan) through a cervical vein by using the same micro-infusion pump that had been used for the mannitol or saline infusion. Thereafter, each animal was immediately decapitated, and its brain was removed and fixed in 10% phosphate-buffered formaldehyde for 3 days. Each fixed brain was cut coronally at the chiasmatic level, and HE-stained paraffin sections of 3-μm thickness were examined. Using an eyepiece micrometer (U-OCMSQ10/10, Olympus, Tokyo, Japan) under $200 \times$ magnification, we counted the numbers of CBSP microvessels (Fig. 2C,D) in all six cerebral cortical layers in the four columns of the left cerebral hemisphere of Face A (Fig. 2A). The number of CBSP microvessels was determined by moving the specimen vertically and medially along four 0.25 mm-wide paths of the cortex, beginning medial to the center point between the rhinal and inter-hemispheric fissures on the HE-stained sections.

To avoid the potential confounding effects of differences in the amount of CBSP microvessels among different animal brains or variations in the thickness of paraffin sections, we also counted the numbers of CBSP microvessels in each of the four columns located in the corresponding positions of the opposite, non-ischemic right cerebral hemisphere. We expressed the number of CBSP microvessels in each column of the ischemic left hemisphere as the ratio as a decimal fraction with respect to the number found in the non-ischemic right hemisphere of each animal.

Area size and mitochondrial number of the end-feet

For the electron-microscopic (EM) study of the control, no-treatment and mannitol groups at 5 h after the final temporary ischemic insult, we fixed the brains of three animals from each group. Transcardiac perfusion for 5 min with a diluted fixative (1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol/L cacodylate buffer) was followed by that for 20 min with more concentrated fixative (4% paraformaldehyde and 5% glutaraldehyde in 0.1 mol/L cacodylate buffer). We obtained two cortical blocks that included all six cortical layers along a 1 mm-wide column medial and lateral to the center point between the rhinal and interhemispheric fissures of the left cerebral cortex on Face A. These blocks were embedded in Epon Ultrathin sections, including the second through the fifth cortical layers, were double-stained with uranyl acetate and lead citrate, and were then examined with an electron microscope (H-9000, Hitachi, Tokyo, Japan) (Fig. 3). Using a computer-assisted digital planimeter, we measured the areas ($μm^2$) of the end-feet around microvessels and counted the numbers of mitochondria in the end-feet. These data were expressed as the average areas of the end-feet and the numbers of end-feet mitochondria in each of the three groups, the data of which were obtained from 88 evenly distributed EM pictures of end-feet taken at 4050- to 10 800-fold magnification. Toluidine blue-stained sections from each Epon block were investigated by LMS.

Osmotic pressure and hematocrit

We obtained blood samples from the jugular vein of each animal in the control (sham-operated) and mannitol groups. Mannitol was administered twice, at 0 and 4 h, after the final 10 min of temporary ischemia; and blood samples were obtained at 0.5, 1, 4, 4.5, 5 and 6 h after the temporary ischemia. The plasma osmotic pressure was measured by using a vapor pressure osmometer (Vapro 5520, Wescor, Inc, UT, USA). The hematocrit levels of each sample were also measured.[12]

Statistical analysis

We statistically analyzed the differences between the time points and groups by using analysis of variance followed by

Table 1 Plasma osmolality and hematocrit in gerbils following intravenous infusion of mannitol

			Time after the temporary ischemia[†]				
	Control	0.5 h	1 h	4 h	4.5 h	5 h	6
Plasma osmolality (mOsm/L)	320.2 ± 2.2	353.1 ± 4.7[‡]	326.4 ± 1.5	320.1 ± 1.8	337.2 ± 2.8[‡]	319.1 ± 1.0	314.0 ± 1.3
Hematocrit (decimal fraction)	0.458 ± 0.005	0.406 ± 0.006[‡]	0.429 ± 0.006	0.424 ± 0.006	0.350 ± 0.014[‡]	0.428 ± 0.007	0.433 ± 0.004

†i.v. injection of 20% Mannitol solution (1.2 g/kg) at 0 and 4 h after the temporary ischemia. ‡Significant difference vs control at $P < 0.01$. Data are the mean ± SE ($n = 9$).

the Bonferroni-Dunn test. All the data in the text, Figure 4 and Table 1 are presented as the averages ± SE. The cut-off for a statistically significant difference was accepted at $P < 0.01$.

RESULTS

Infarct size

A focal cerebral cortical infarction developed on Face A at 72 h after the final temporary ischemia in the no-treatment group (Fig. 1A) and was remarkably reduced in size at 120 h in the mannitol group (Fig. 1B). The average ratio as a decimal fraction of the infarct area to the total area of the cerebral cortex was remarkably reduced at 72 h after the ischemia, from 0.205 ± 0.016 in the no-treatment group (Figs 1C,4A) to 0.021 ± 0.003 in the mannitol group (Figs 1E,4A). At 120 h after the final ischemia, the ratios had dropped to 0.122 ± 0.011 in the no-treatment group (Figs 1D,4A) and to 0.019 ± 0.004 in the mannitol group (Figs 1F,4A), due to a reduction in the edema in the neuropil. However, the difference between the ratios of the mannitol group at 72 and 120 h was not significant. The ratio for the saline group was reduced from 0.207 ± 0.018 at 72 h after the temporary ischemia to 0.116 ± 0.011 at 120 h after it, but there was no significant difference between each corresponding value in the no-treatment group (Fig. 4A).

Number of patent CBSP microvessels

In the present animal model, cerebral cortical infarction developed in the region of the maturing DSNN seen in the sections cut coronally at the chiasmatic level (Face A, Fig. 2A). However, only DSNN maturation occurred in the cerebral cortex in the corresponding sections made at the infundibular level (Face B, Fig. 2B). In the cerebral cortices of the HE-stained coronal sections of Face A, the number of the CBSP microvessels was reduced in the no-treatment group compared with that for the mannitol group at 5 h after the temporary ischemia (Fig. 2C,D). The average ratio as a decimal fraction of the number of CBSP microvessels in the left compared with that in the right

cerebral cortices was calculated for each group at each time period (Fig. 4B, a part of the data is quoted from Ito *et al.*[7]). In the no-treatment group, there was no statistical difference between the decimal fraction ratio 0.931 ± 0.036 for the control and 0.865 ± 0.048 at 0.5 h after the temporary ischemia. These ratios then decreased significantly to 0.443 ± 0.018, 0.359 ± 0.020 and 0.324 ± 0.028 at 3, 5 and 8 h, respectively, after the final temporary ischemia, due to microvasculogenic secondary focal cerebral ischemia. Thereafter, the ratio increased to 0.625 ± 0.057 and 0.762 ± 0.071 at 12 and 24 h, respectively, after the final temporary ischemia, due to necrosis of the astrocytes and their end-feet in the developing infarction.[7] In the mannitol groups, the ratios were 0.978 ± 0.048, 0.931 ± 0.034 and 0.897 ± 0.025 at 3, 5 and 8 h, respectively, after the temporary ischemia, and each ratio was significantly higher than the corresponding one for the no-treatment group, but there were no statistical differences among control and manitol groups at 3, 5 and 8 h after the temporary ischemia.

Area and mitochondrial number of the end-feet

Obstructed microvessels compressed by the swollen astrocytic end-feet and microvascular stasis were observed by LMS at 5 h after the temporary ischemia in Toluidine blue-stained Epon sections from the no-treatment group, whereas only slightly swollen astrocytic end-feet were observed in the mannitol group (Fig. 2E,F, respectively). At 5 h after the temporary ischemia in the no-treatment group, the EM study revealed remarkably swollen astrocytic end-feet, the presence of obstructed microvessels compressed by the surrounding swollen astrocytic end-feet, and mitochondria in the end-feet that were slightly enlarged and degenerated with disoriented cristae with cavitation in the matrix (Fig. 3A–F). Evidence of stasis, that is, the presence of stagnated plasma, erythrocytes, polymorphnuclear leucocytes and platelets, was observed in the microvessels surrounded by swollen astrocytic end-feet (Fig. 3D–F). The swelling of astrocytic end-feet was remarkably reduced in the mannitol group. Mitochondria in the end-feet were slightly degenerated with disoriented cristae and a cavitated matrix, but these changes were less so than those in the no-treatment group
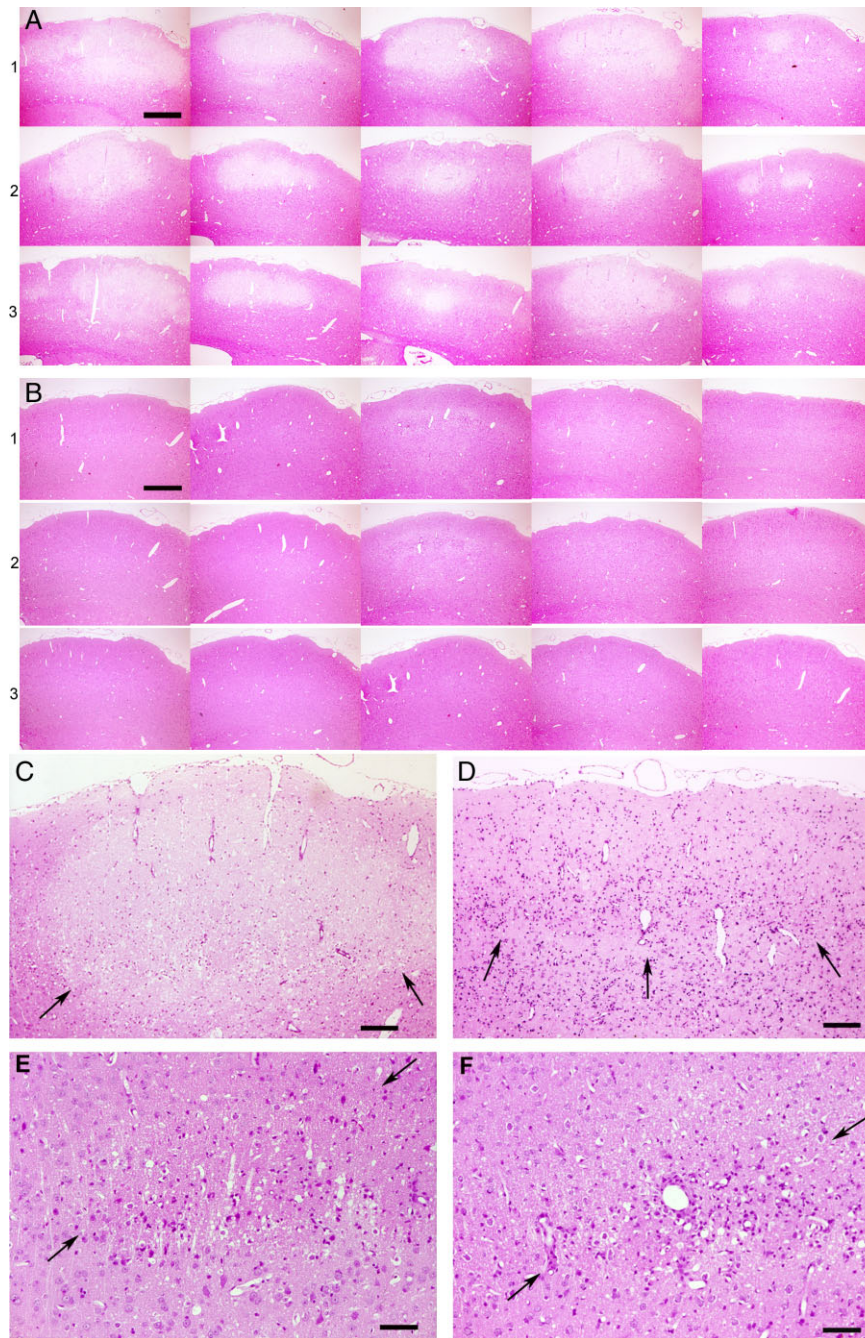
Fig. 1 Light microscopic findings of focal infarction in the left cerebral cortex. Coronally cut paraffin sections were prepared at the chiasmatic level (Face A); HE staining. (A,B) Four-power magnification of focal cortical infarction in each of three paraffin sections (1, 2, 3) cut at 400-μm intervals from each of five animals; bar = 0.72 mm. (A) Seventy-two hours after temporary ischemia in the no-treatment group, well-circumscribed infarctions are found in the cerebral cortex. (B) One-hundred and twenty hours after temporary ischemia in the mannitol group. No infarctions are discerned at this magnification. (C–F) Higher power magnification of focal cortical infarction: ×50 (C–D) and ×100 (E–F). Arrows indicate areas of the focal infarction. Condensed, dead neurons with eosinophilic cytoplasm with or without pyknotic or karyorrhectic nuclei are scattered throughout the spongy edematous necrotic neuropil. The swollen astrocytic processes are arranged in parallel. Thick-walled microvessels that had proliferated are visible. There is evidence of infiltration by monocytes and macrophages, as well as their mitotic figures. (C) Seventy-two hours after ischemia in the no-treatment group; bar = 0.22 mm. (D) One-hundred and twenty hours after ischemia in the no-treatment group; bar = 0.15 mm. The numbers of swollen astrocytic processes are reduced, indicating decreased edema. (E) Seventy-two hours after ischemia in the mannitol group; bar = 85 μm. A small focal infarction is present in the third cortical layer. (F) One-hundred and twenty hours after ischemia in the mannitol group; bar = 85 μm. A small focal infarction is visible in the third cortical layer. In the spongy neuropil, the edema is reduced, as is the number of condensed, dead neurons.

(Fig. 3H,I). No swelling of the end-feet was found in the sham-operated control group (Fig. 3G).

At 5 h after the temporary ischemia, the average measured areas of the astrocytic end-feet were 6.0 ± 0.64 μm², 73.3 ± 8.11 μm² and 16.9 ± 1.92 μm² in the control, no-treatment and mannitol groups, respectively (Fig. 4C). However, there was no statistical difference between the control and mannitol groups. The average number of mitochondria in the end-feet was 8.04 ± 1.09, 8.60 ± 1.93 and 6.90 ± 0.82 in the control, no-treatment and mannitol groups, respectively; and no statistical differences were found among them (Fig. 4D).

Osmotic pressure and hematocrit

The plasma osmotic pressure (mOsm/L) in the mannitol group was 353.1 ± 4.7 at 0.5 h after the first mannitol infusion, which was relatively higher than the value of 320.2 ± 2.2 in the control group. Subsequently, it decreased to 326.4 ± 1.5 at 1 h after the temporary ischemia. Prior to the second mannitol infusion, the plasma osmotic pressure in the mannitol group was 320.1 ± 1.8 at 4 h after the temporary ischemia. Half an hour after the second mannitol infusion, this value increased to 337.2 ± 2.8 at 4.5 h after the temporary ischemia. Then it decreased to 319.1 ± 1.0 and

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Fig. 2 Light microscopic findings. (A) Coronal section at the chiasmatic level (Face A). The four 0.25-mm-wide cortical columns that were counted are indicated. "1/2" indicates the center point between the rhinal (RF) and interhemispheric fissures (IF) along the cortical surface; PAS staining. (B) Coronal section at the infundibular level (Face B). No infarcts developed in the area of the cerebral cortex where the maturation of neuronal necrosis is found; PAS staining. (C) Five hours after the temporary ischemia in the no-treatment group. The black arrows indicate carbon-black-suspension-perfused (CBSP) microvessels, and the blue arrows, non-perfused and/or stagnated microvessels. HE staining; bar = 38 μm. (D) Five hours after the temporary ischemia in the mannitol group. The arrows are defined in "C"; HE staining; bar = 38 μm. The number of CBSP microvessels has increased. (E) Five hours after the temporary ischemia in the no-treatment group. Signs of stasis (arrows) and obstructed microvessels in the swollen astrocytic end-feet (arrowheads) are observed. Toluidine blue-stained Epon section; bar = 12.2 μm. (F) Five hours after the temporary ischemia in the mannitol group. Microvessels are surrounded by slightly swollen astrocytic end-feet (arrows). Toluidine blue-stained Epon section; bar = 12.2 μm.
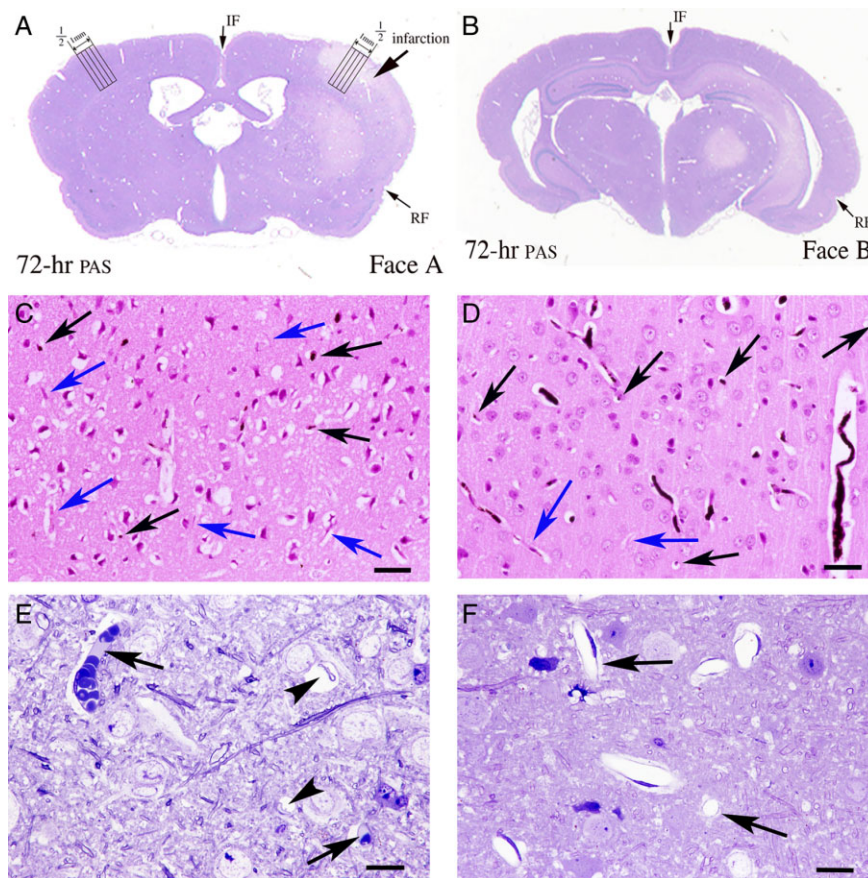
314.0 ± 1.3 at 5 and 6 h, respectively, after the temporary ischemia. The changes in hematocrit were inversely proportional to the changes in the osmotic pressure[12] (Table 1).

DISCUSSION

Until the early 1970s, it had been thought that neurons died quickly after even a short period of ischemia. Using a stroke-positive unilateral carotid occlusion model of Mongolian gerbils,[14–16] we found in 1975 that rapid death of neurons does not occur soon after a temporary ischemic insult. Rather, the intensity of the ischemic injury and speed of its appearances are directly related to the duration of ischemia; thus, we referred to these changes as the "maturation phenomenon."[17] The maturing ischemic damage after recirculation to the ischemic brain had previously been considered as being due to microvascular dysfunction/inflammation and mitochondrial damage that interact to evoke secondary tissue damage in the reperfusion tissue.[18–22]

In our earlier study, assessed by perfusion with carbon-black suspension and/or C[14]-antipyrine autoradiography in a stroke-positive model of temporary unilateral carotid occlusion lasting 30 min or 6 h, we found that the no-reflow phenomenon disappeared in < 0.5 min and < 10 min, respectively, after the beginning of recirculation, and that then the blood flow was completely restored.[6,23] In our last study, using the same model as in the present study, we found that on coronal sections prepared at the chiasmatic level (Face A Fig. 2A), 97.5 ± 4.16% of the microvessels were patent, as evidenced by perfusion with carbon-black suspension, immediately after reperfusion following temporary cerebral ischemia.[7,12] Also we found that secondary focal ischemia was induced by microvascular obstruction and stasis due to compression by the swollen astrocytic end-feet that occurred during the period of 3 to 8 h after the start of reperfusion following temporary cerebral ischemia and that this cortical focal infarction developed between 12 and 24 h after the temporary ischemia.[7] Furthermore, in the present animal model, we did not observe development of cortical focal infarction in the maturing DSNN on the surface cut coronally at the infundibular level (Face B Fig. 2B)[24] until 12[25] or 24 weeks[26] after the temporary ischemia. These findings also suggested that the development of the cortical focal infarction had not occurred continuously following the maturing neuronal injuries, but had been induced by the additional microvasculogenic secondary focal cerebral ischemia.[3,7]
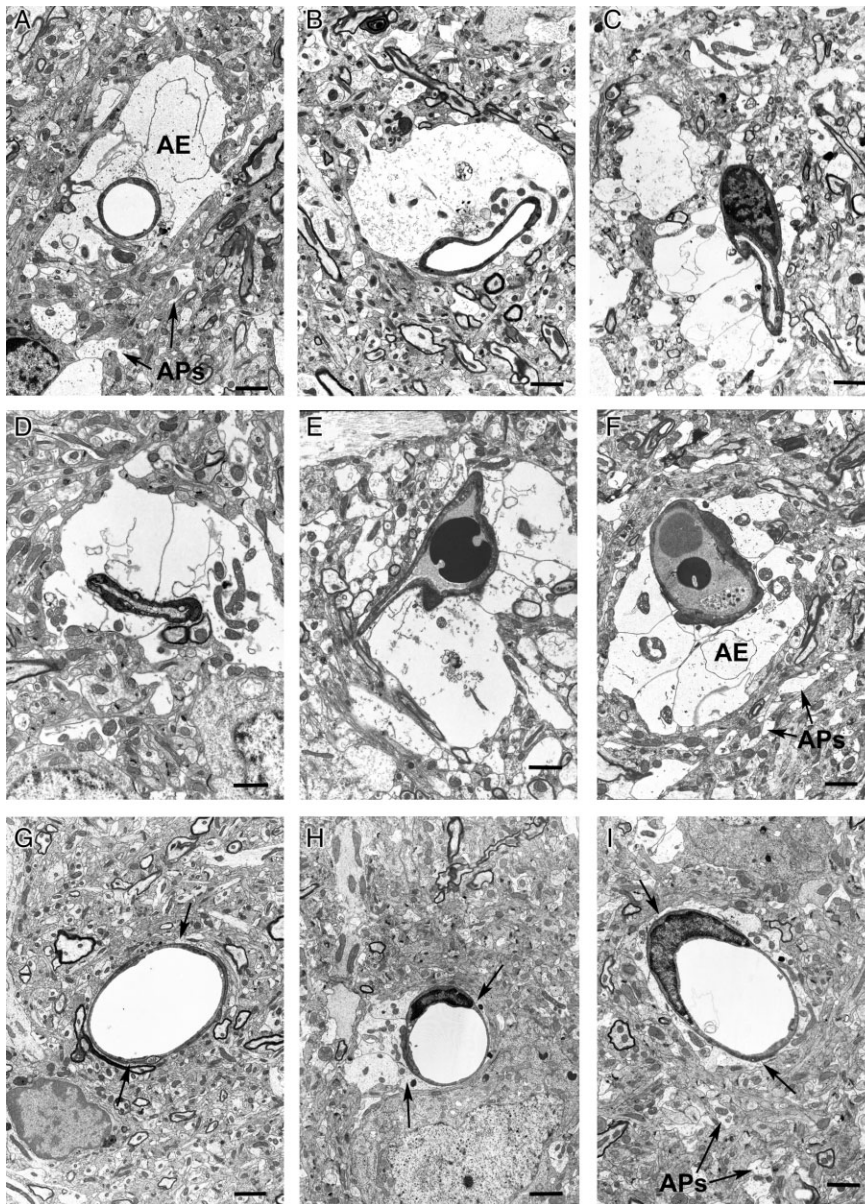
Fig. 3 Electron microscopic findings at 5 h after the temporary ischemia; bar = 2.09 μm. AE and arrows indicate astrocytic end-feet. APs indicate swollen astrocytic processes. (A–F) No-treatment group. Mitochondria in the end-feet are slightly enlarged and degenerated with disoriented cristae and cavitation in the matrix. Extraordinarily swollen astrocytic end-feet compared with the swollen astrocytic processes in the surrounding neuropil are observed around the microvessels. (B–F) Remarkably swollen astrocytic end-feet have compressed microvessels. (D–F) Microvascular stasis is seen with erythrocytes, neutrophils and platelets in the stagnated plasma. (G) Microvessel of a sham-operated control animal. Astrocytic end-feet are very thin and surround the entire vessel (arrows). (H–I) Mannitol group. Slightly swollen astrocytic end-feet are observed surrounding microvessels (arrows). Mitochondria in the end-feet are slightly degenerated with disoriented cristae and cavitation in the matrix but less so than in the no-treatment group.

In the present study, we found that mannitol infusion started immediately after the start of reperfusion reduced the swelling of the astrocytic end-feet, prevented the microvascular compression and stasis caused by them, and remarkably reduced the infarct size.

The twofold increase in the plasma osmolality and the decrease in the hematocrit continued for less than 1 h after each of the two mannitol infusions. Therefore, the reduction in the swelling of the astrocytic end-feet in the present study can be considered to have been due not only to the water shift produced by the osmotic difference, but also to the recovery from a reperfusion injury by the removal of metabolic disturbances in the astrocytes and/or their end-feet. Five hours after the blood flow had resumed, rapid swelling of the astrocytic end-feet in the no-treatment group led to a volume ratio of 42.6 times that of the control; whereas in the mannitol group, this value was 4.7 times that of the control (Fig. 3, Table 2).[7] The mechanism responsible for the rapid swelling of the astrocytic end-feet, as well that for suppression of the swelling by the mannitol infusion immediately after the restitution of the blood flow is unknown. However, these changes in the astrocytic end-feet, together with the site of the threshold for the focal cortical infarction induced by the microvasculogenic secondary focal cerebral ischemic injury, would seem to be an important pathological target to prevent the development of the focal infarction after reperfusion. Aquaporin function might be also involved in these mechanisms[27,28] and further study is mandatory. Because there was no significant effect of saline infusion on
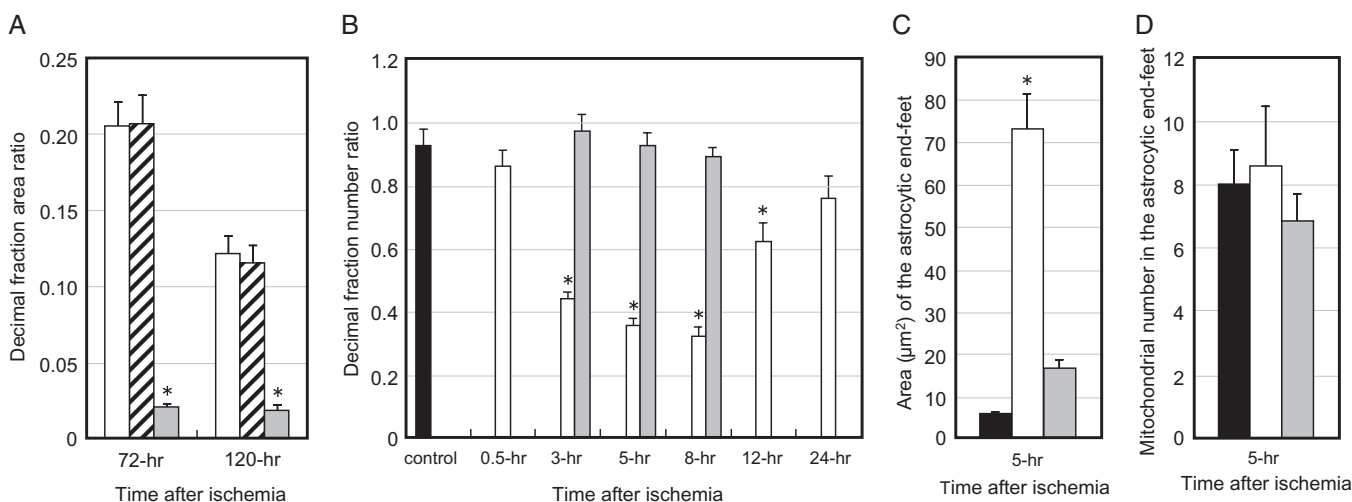
Fig. 4 In each bar graph, A–D, the black bars indicate the control; white bars, no-treatment; gray bars, mannitol treatment; and bars with oblique lines, saline groups. A part of the data for the no-treatment group is quoted from Ito *et al.*[7] (A) Area ratio as a decimal fraction of the infarction in the cerebral cortex (*n* = 15 for each group). *P < 0.01 versus the no-treatment or saline group at 72 and 120 h after the temporary ischemia. There is neither a significant difference between the no-treatment and saline groups, nor one between the mannitol groups at 72 and 120 h after the ischemia. (B) Number ratio as a decimal fraction of the carbon-black-suspension-perfused microvessels in the left cerebral hemisphere to those in the right. *P < 0.01 (*n* = 12) versus the control. There is no significant difference between the control and the no-treatment group at 0.5 or 24 h, nor between the control and the mannitol group at 3, 5 or 8 h. (C) Area (μm²) of the astrocytic end-feet at 5 h. *P < 0.01 (*n* = 32) versus the control (*n* = 24) or mannitol group (*n* = 32) for the area of the end-feet. (D) Numbers of mitochondria in the end-feet at 5 h. There is no significant difference between the control and mannitol or no-treatment group in terms of the numbers of mitochondria in the astrocytic end-feet.

Table 2 Ratios of the volume of various parts of astrocytes to those of the control

	Time after ischemia[†]			Time after ischemia[‡]
	0.5 h	5 h	5 h (mannitol)	5 h
Astrocytic cytoplasm	4.0	6.8	–	2.6
Astrocytic processes	2.9	4.3	–	2.5
Astrocytic end-feet	9.6	42.6	4.7	14.3

Data were calculate from References 14 and 15 and the present study. †Coronally cut at chiasmatic level where focal cerebral cortical infarction developed in the area of maturing neuronal necrosis. ‡Coronally cut at infundibular level where only neuronal necrosis matured.

the infarct size (Fig. 4A), the slight decrease in hematocrit (Table 1) would not have influenced the microvascular circulation.

The reason for this is described in Benedek *et al.*,[29] that formation of formazan from 2,3,5-triphenyltetrazolium chloride (TTC) can depend on both the staining method (*in vitro* and *in vivo*) and the metabolic burdens of the brain tissue (intensity of and recovery from ischemia). These caused uncertainties in the volume of ischemia which induced brain injury measured by TTC staining. To measure accurate size of infarction, we have applied the histopathological measurement under the microscopy instead of TTC.[29]

We calculated the area ratio of infarction to the area of the cerebral cortex for each coronal section and then obtained the average value to avoid introducing confounding differences in the sectioning and spreading of each section. The ratio of the normal blood flow to the blood

flow necessary to induce an infarction remains approximately constant at 3:1 across species.[1]

As the gerbil brain is small, histologically comparative measurement of regional cerebral blood flow (rCBF) using [14C] antipyrine autoradiography[23] as well as [3H] nicotine liquid scintillation method[14] are inappropriate. In the present study, same as the previous study[7] as well as the permanent ischemia model by Little,[12] we counted CBSP patent micro-vessels and compared them with the histopathological findings under the LMS.

In the present study, the decimal fraction ratio for the number of CBSP microvessels in the left ischemic hemisphere to that in the right non-ischemic hemisphere was 0.931 ± 0.036 in the control, and it decreased from 0.865 ± 0.048 at 0.5 h to 0.324 ± 0.028 at 8 h after the temporary ischemia in the no-treatment group due to the microvasculogenic secondary focal cerebral ischemia. Then this ratio increased to 0.625 ± 0.198 and 0.762 ± 0.246 at 12

and 24 h, respectively, after the temporary ischemia by release from the microvascular compression due to necrosis of astrocytes and their end-feet in the developing focal infarction,[7] whereas, in the mannitol group this ratio did not drop significantly at 3, 5 or 8 h after temporary ischemia; and there was no significant difference between it at these times and the control value (Fig. 4B). Therefore, it is inconceivable that metabolically progressed infarction induced secondary microvascular disturbances.

Recent MRI studies have revealed the presence of ischemic lesions following stenting or endarterectomy for symptomatic carotid stenosis,[30] and so the swelling of the astrocytic end-feet and the compressive obstruction of microvessels may occur soon after the reopening of an obstructed major vessel by tissue plasminogen activator (tPA) administration and/or arterial stenting. Because the safety of mannitol use is well established, mannitol infusion immediately after the start of reperfusion could be used as an adjuvant to tPA and stenting therapy and should result in better prognoses by protecting against the development of a cerebral infarction.

ACKNOWLEDGMENTS

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