

Effect of Nitrous Oxide on Neuronal Damage and Extracellular Glutamate Concentration as a Function of Mild, Moderate, or Severe Ischemia in Halothane-anesthetized Gerbils

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Background: The effect of nitrous oxide on ischemic neuronal damage was quantitatively evaluated by use of logistic regression curves.

Methods: Seventy-two gerbils were anesthetized with 1% halothane and randomly assigned to receive 70% nitrous oxide or 70% nitrogen. Forebrain ischemia was performed for 3, 5, or 7 min, and direct-current potential in the hippocampal CA1 region was recorded. Histologic outcome was evaluated 5 days later. Relations of neuronal damage with ischemic duration and duration of ischemic depolarization were determined by logistic regression curves. In some animals, extracellular glutamate concentration was measured every 60 s during forebrain ischemia.

Results: Nitrous oxide increased neuronal damage only with 5 min of ischemia (nitrous oxide vs. nitrogen: 78.5 ± 23.0 vs. $37.3 \pm 12.2\%$; $P < 0.01$). The percentages of neuronal damage with 3 and 7 min of ischemia were not different with or without nitrous oxide. Logistic regression curves indicated that nitrous oxide significantly increased neuronal damage during the period from 3.07 to 6.63 min of ischemia. Logistic regression curves also indicated that nitrous oxide increased neuronal damage in the condition of the same duration of ischemic depolarization. Nitrous oxide shortened the ischemic duration necessary for causing 50% neuronal damage by 0.82 min. Dynamic change in extracellular glutamate concentration was not different (mean maximum dialysate glutamate concentration: 4.29 ± 3.09 vs. $4.63 \pm 1.83 \mu\text{M}$).

Conclusion: Administration of nitrous oxide caused an increase in ischemic neuronal damage, but a significant adverse effect was observed with a limited range of ischemic intervals.

NITROUS oxide has been used in general anesthesia for more than 150 yr. Nitrous oxide can provide a certain level of intraoperative analgesia and stabilization of arterial blood pressure, and the low value of blood gas partition coefficient of nitrous oxide enables rapid recovery and early postoperative neurologic assessment.¹

Although several studies had been conducted, a definite conclusion regarding the effect of nitrous oxide on ischemic neuronal damage has not been obtained. Baughman *et al.*² reported that nitrous oxide attenuated the neuroprotective effect of isoflurane in rats, and Hartung *et al.*³ reported that nitrous oxide shortened the survival time in mice. On the other hand, Warner *et al.*⁴ and Yokoo *et al.*⁵ reported that nitrous oxide did not affect the neurologic and histologic outcomes in rats, and David *et al.*⁶ reported that administration of nitrous oxide immediately after ischemia for 3 h reduced neuronal damage in rats.

Reduction of cerebral blood flow during ischemia impairs the delivery of oxygen and glucose. With the depletion of oxygen and glucose, membrane potential is lost and neuronal cells are depolarized. Excitatory amino acids (especially glutamate) are then released from the presynaptic folliculus into the extracellular space, and reuptake of excitatory amino acids is also inhibited by the loss of sodium gradient across the cell membrane. Increase in extracellular glutamate activates ionotropic and metabotropic glutamate receptors. As a consequence, calcium ions flow into the postsynaptic neurons, triggering the irreversible process leading to neuronal damage.^{7,8} It is known that onset time of ischemic depolarization and duration of ischemic depolarization affect the degree of neuronal damage,^{9,10} and ischemia-induced increase in extracellular glutamate concentration contributes to excitotoxic neuronal damage.¹¹ However, to the best of our knowledge, the effects of nitrous oxide during cerebral ischemia on ischemic depolarization and extracellular glutamate concentration have not been investigated.

Therefore, in the current study, the effects of nitrous oxide at a clinically relevant concentration on ischemic neuronal damage were quantitatively evaluated. For this purpose, cerebral ischemia of three intensities was performed in the same ischemia model, and relations of neuronal damage with ischemic duration and duration of ischemic depolarization were determined by use of logistic regression curves. At the same time, ischemic duration necessary for causing 50% neuronal damage was determined. Furthermore, the effect of nitrous oxide on dynamic change in extracellular glutamate concentration during cerebral ischemia was determined.

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Materials and Methods

Animals

A total of 72 male Mongolian gerbils (Charles River Japan, Yokohama, Japan) weighing 70.6 ± 5.2 g were used. The animals had free access to water and were fed *ad libitum* before the experiments. All experiments were performed in accordance with the National Institutes of Health animal care guidelines and were approved by the Animal Research Control Committee of Okayama University Medical School, Okayama City, Okayama, Japan.

Evaluation of the Relations of Neuronal Damage with Ischemic Duration and Duration of Ischemic Depolarization

Forty-eight male Mongolian gerbils were used in this experiment. The animals were randomly assigned to a group receiving nitrous oxide (nitrous oxide group, $n = 24$) and a group receiving nitrogen (nitrogen group, $n = 24$). Anesthesia was induced with 4% halothane in oxygen and maintained with 1% halothane in 30% oxygen balanced with 70% nitrous oxide (nitrous oxide group) or 70% nitrogen (nitrogen group) under spontaneous ventilation. Because minimum alveolar concentration (MAC) of halothane has been reported to range between 0.88 and 1.26 vol% in rodents,¹²⁻¹⁴ 1% halothane was considered to be approximately 1 MAC in the current study. Bilateral common carotid arteries were exposed, and a silicon tube for initiation of ischemia (0.3 mm in diameter) was placed around each of these arteries. After head placement in a stereotaxic apparatus (Narishige, Tokyo, Japan), an electroencephalogram was monitored with needle electrodes placed subcutaneously in the left frontal region. After making bar holes in bilateral temporal bones, two borosilicate glass electrodes (tip diameter of $<5 \mu\text{m}$) were placed in the bilateral hippocampal CA1 regions according to the brain atlas (2 mm posterior to the bregma, 1.5 mm bilateral from the sagittal line, and 1 mm below the cortical surface).¹⁵ A laser-Doppler flow probe (ALF 2100; Advance, Tokyo, Japan) was placed on the surface of the right temporal bone for continuous monitoring of regional cerebral blood flow.

After a 30-min stabilization period after surgical preparation, forebrain ischemia was initiated by occlusion of bilateral common carotid arteries with plumbs (each weighing 4 g) for 3, 5, or 7 min ($n = 8$ in the two groups for each duration). The ischemia and reperfusion were confirmed by the sudden decrease and rapid increase in regional cerebral blood flow on a laser-Doppler flow meter. Changes in direct-current (DC) potentials, electroencephalograms, and regional cerebral blood flow were recorded and analyzed using an analog-digital system (Axo-Scope and Digidata 1200 B; Axon Instruments, Foster, CA) from 5 min before the ischemia to 45 min after the initiation of ischemia. Changes in DC potentials

were assessed by measuring onset time (from the initiation of ischemia to a sudden negative shift of DC potential), recovery time (from the initiation of recirculation to 80% recovery from maximal DC deflection), and duration of ischemic depolarization (from the start of the sudden negative shift of DC potential to 80% recovery from maximal DC deflection). Because Dirnagl *et al.*¹⁶ reported that data obtained by using a laser-Doppler flow meter only provide accurate information on change in regional cerebral blood flow, the percent change in regional cerebral blood flow was expressed in the current study.

During the observation period, administration of nitrous oxide or nitrogen was continued. Brain surface temperature was maintained at $37.0^\circ \pm 0.5^\circ\text{C}$ with a gentle flow of warmed saline ($38.0^\circ \pm 0.5^\circ\text{C}$, monitored and controlled) into a polyethylene cylinder (5 mm in height, 13 mm in ID) placed on the skull surface. Rectal temperature was maintained with a heated-water blanket and an infrared lamp at $37.0^\circ \pm 0.5^\circ\text{C}$. Because Kuroiwa *et al.*¹⁷ reported that the possibility of neuronal death caused by postischemic hyperthermia could be excluded by maintenance of normothermia after 85 min after reperfusion, brain surface and rectal temperatures were controlled from 30 min before the ischemia to 90 min after the reperfusion.

After a 5-day survival period, all animals were anesthetized with 4% halothane in oxygen and perfused with heparinized physiologic saline (20 U/ml) and 6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The areas in which DC potential had been recorded were marked by using a 27-gauge needle with blue ink on the tip of the needle before enucleating the brains. After brain removal and paraffin embedding, tissue including the bilateral hippocampal CA1 regions (the area marked with blue ink) was sectioned coronally (5 μm in thickness). The sections were stained with hematoxylin and eosin. The number of injured pyramidal neurons in the bilateral hippocampal CA1 regions was counted by an observer who was blinded to this study. The areas in which DC potential had been recorded were identified by finding the ink spot or the mark made with the needle with 40-power magnification. The area was enlarged to 400-power magnification, and the numbers of both damaged and intact pyramidal neurons in bilateral hippocampal CA1 regions were counted. The percentages of neuronal damage in the two groups were calculated as damaged neurons/total neurons $\times 100$ in the visual field. In the current study, pyramidal neurons showing aggregated chromatin in the nucleus, shrinkage, or eosinophilic staining in the cytoplasm were considered to be injured.

Dose-reaction curves evaluating acute drug toxicity in toxicology are usually expressed by the use of probit curves. In the current study, the relations of neuronal damage with ischemic duration and duration of ischemic

depolarization were determined by logistic regression curves (probit curves) as dose–reaction curves. Ischemic duration or duration of ischemic depolarization was represented on the x-axis, and neuronal damage was represented on the y-axis. The y-axis was converted to probit transformation, and regression lines in the two groups were drawn. At the same time, 95% confidence intervals were also drawn. Finally, the y-axis was returned to percent change. These regression curves were drawn by using data-analysis software (Origin Pro 7.5J; OriginLab Corporation, Northampton, MA). A probit curve, which expresses the probability of occurrence, is used to search for the median lethal dose in toxicology. Therefore, in this study, ischemic durations necessary for causing 50% neuronal damage (P_{50} -ischemia) in both groups were determined from logistic regression curves.

Because bleeding during arterial catheterization or blood sampling (100 μ l) decreased arterial pressure in gerbils and it may affect the neuronal damage after forebrain ischemia, we evaluated physiologic variables in another set of animals. Ten animals were randomly assigned to a group receiving nitrous oxide (nitrous oxide group, $n = 5$) or a group receiving nitrogen (nitrogen group, $n = 5$). A polyethylene catheter (PE-10; IMI, Tokyo, Japan) was inserted into the right femoral artery for continuous monitoring of mean arterial pressure. Bilateral common carotid arteries were exposed, and a silicon tube for initiation of ischemia was placed around each of these arteries. After head placement in a stereotaxic apparatus, 5 min of forebrain ischemia was performed by the same procedure as that used for the measurement of DC potential. Mean arterial blood pressure was recorded and analyzed using an analog–digital system (Axo-Scope and Digidata 1200 B) from 5 min before the ischemia to 20 min after the initiation of ischemia. Arterial blood gas analysis (pH, arterial oxygen tension, arterial carbon dioxide tension, hydrogen carbonate ions, blood glucose, and hematocrit) was performed before the ischemia (i-STAT; Fuso, Osaka, Japan).

Statistical Analyses. All values are expressed as mean \pm SD. Data for physiologic values and onset time were analyzed by Student *t* test. The changes in cerebral blood flow on a laser-Doppler flowmeter and mean arterial blood pressure were analyzed by two-way analysis of variance (groups *vs.* time). Other values were analyzed with analysis of variance followed by Scheffé test for multiple comparisons. A level of $P < 0.05$ was considered to be significant in all statistical tests.

Measurement of Dynamic Changes in Extracellular Glutamate Concentration

Fourteen male Mongolian gerbils were used in this experiment. The animals were randomly assigned to a group receiving nitrous oxide (nitrous oxide group, $n = 7$) or a group receiving nitrogen (nitrogen group, $n = 7$). Concentrations of oxygen, nitrous oxide or nitrogen,

and halothane were the same as those in the first experiment. The preparative surgery until head placement was also the same as that in the first experiment. After making a bar hole in the right temporal bone, a microdialysis probe (membrane length of 1.5 mm, molecular weight cutoff of 50,000, OD of 220 μ m; A-I-015, Eicom, Kyoto, Japan) was inserted instead of the borosilicate glass electrode in the area that mostly reflected the right hippocampal CA1 region (2 mm posterior to the bregma, 1.5 mm lateral from the sagittal line, and 2 mm below the cortical surface). Before the experiment, the relative recovery rate of each probe was determined by performing microdialysis in a glutamate standard solution (100 μ M). The probes were perfused with Ringer's solution at 2 μ l/min using an infusion pump (ESP-32; Eicom). The bias of increase in extracellular glutamate concentration caused by mechanical injury during probe insertion disappeared within 30 min. Therefore, after a 60-min stabilization period after the microdialysis probe insertion, 5 min of forebrain ischemia was performed by the same procedure as that used in the first experiment. The dialysate was automatically collected every 60 s using a fraction collector (EFC-82; Eicom) from 5 min before to 30 min after the reperfusion.

Quantification of glutamate was performed by high-power liquid chromatography with computerized control (Nanospace Syscon 21; Shiseido, Tokyo, Japan). Because such a small amount of sample (2 μ l each) cannot be injected into the chromatography column, collected dialysates were diluted by adding Ringer's solution (4 μ l each). Diluted solutions (each 5 μ l) were used to separate glutamate using an isolation column (4.6 \times 150 mm, GU-GEL; Eicom). Isolated glutamate was converted to hydrogen peroxide by glutamate oxidase packed in an enzyme immobilized column (E-ENZYMYPAC; Eicom). For electrochemical detection, oxidative potential was applied to a platinum electrode at 450 mV *versus* an Ag/AgCl reference electrode. The mobile phase (50 mM ammonium chloride) was pumped with a microvolume plunger system (Nanospace SI-2 3001; Shiseido) at a rate of 400 μ l/min. Corrected maximum extracellular glutamate concentration was calculated from the dialysate glutamate concentrations divided by relative recovery rate of each probe.

During the experimental period, administration of nitrous oxide or nitrogen was continued. Brain surface and rectal temperatures were maintained at $37.0^\circ \pm 0.5^\circ\text{C}$ as done in the first experiment from 30 min before the ischemia to the end of the experiment.

Statistical Analyses. Values are expressed as mean \pm SD. Statistic differences were evaluated by two-way analysis of variance (groups *vs.* time). To determine whether ischemia had any effect on the glutamate concentration, paired *t* tests were used to compare basal (5 min before the ischemia) *versus* peak glutamate concentrations in the two groups. Statistical differences in dialysate and

Table 1. Physiologic Values and Changes in Mean Arterial Pressure

	Nitrous Oxide Group (n = 5)	Nitrogen Group (n = 5)
Arterial pH	7.34 ± 0.04	7.35 ± 0.02
Paco ₂ , mm Hg	45.6 ± 3.7	44.6 ± 3.1
PaO ₂ , mm Hg	107.0 ± 8.5	99.0 ± 9.1
HCO ₃ , mm	24.4 ± 2.3	24.5 ± 1.7
Blood glucose, mg/dl	171.2 ± 29.7	163.2 ± 20.8
Hematocrit, %	48.2 ± 1.8	47.4 ± 1.3
Change in MAP, mm Hg		
Before ischemia	72.7 ± 3.8	74.2 ± 5.8
During ischemia	97.3 ± 9.4	89.2 ± 9.1
15 min after ischemia	84.7 ± 5.1	82.5 ± 4.1

Values are expressed as mean ± SD. Physiologic values and changes in mean arterial pressure (MAP) in the nitrous oxide group and nitrogen group were not different.

During ischemia = 2 min after the initiation of ischemia; HCO₃ = hydrogen carbonate ions; Paco₂ = arterial carbon dioxide tension; PaO₂ = arterial oxygen tension.

corrected maximum glutamate concentrations in the two groups were analyzed by Mann-Whitney U test. A level of $P < 0.05$ was considered to be significant.

Results

All animals in the two groups in which forebrain ischemia was performed for 3, 5, or 7 min were able to maintain their position symmetrically, walk, and eat food after the ischemia, and they all survived for 5 days after the ischemia.

Results of arterial blood gas analysis are shown in table 1. Blood glucose values in the two groups were higher than the normal range, and other values were within the normal ranges. There were no significant differences between the nitrous oxide group and nitrogen group. The changes in mean arterial pressure (table 1) and regional cerebral blood flow determined by a laser-Doppler flowmeter (rate of changes from the values before ischemia = 100; table 2) were not significantly different between the nitrous oxide group and nitrogen group. The data of electroencephalogram and power amplitude of electroencephalogram are shown in figure 1. There was no significant difference between the two groups with any frequency with multiple comparisons.

Table 2. Changes in Regional Cerebral Blood Flow by Laser-Doppler

	Nitrous Oxide Group (n = 24)	Nitrogen Group (n = 24)
Before ischemia	100	100
During ischemia	9.9 ± 6.6	7.9 ± 3.0
5 min after ischemia	93.2 ± 38.0	98.7 ± 47.1

Values are expressed as mean ± SD. Values before ischemia = 100. Changes in regional cerebral blood flow in the nitrous oxide group and nitrogen group were not different.

During ischemia = 2 min after the initiation of ischemia.

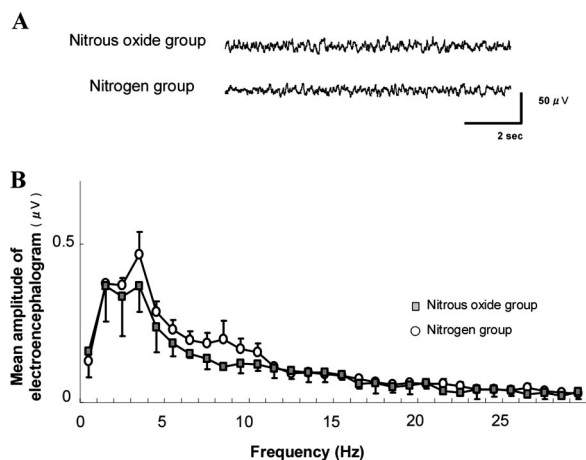


Fig. 1. Plane data of electroencephalography (A) and power amplitude of electroencephalography (B). Power amplitudes in the nitrous oxide group (rectangles) and nitrogen group (circles) were not significantly different with any frequency with multiple comparisons.

The variables of DC potential are shown in table 3. Onset time of ischemic depolarization was significantly shorter in the nitrous oxide group (1.62 ± 0.41 min) than in the nitrogen group (1.90 ± 0.37 min) ($P < 0.01$). There was no significant difference in recovery time and duration of ischemic depolarization between the two groups with any ischemic duration.

The variables of neuronal damage are shown in table 3. The percentage of neuronal damage with 5 min of ischemia was significantly higher in the nitrous oxide group ($78.5 \pm 23.0\%$) than in the nitrogen group ($37.3 \pm 12.2\%$) ($P < 0.01$). Statistical power between the nitrous oxide group and nitrogen group with 5 min of

Table 3. Variables of Direct-current Potential and Neuronal Damage

	Nitrous Oxide Group (n = 24)	Nitrogen Group (n = 24)
Onset time, min	1.62 ± 0.41*	1.90 ± 0.37
Recovery time, min (n = 8 for each duration)		
3 min of ischemia	1.71 ± 0.85	1.60 ± 0.59
5 min of ischemia	2.97 ± 1.10	2.88 ± 0.65
7 min of ischemia	3.76 ± 1.88	4.02 ± 1.12
Duration of ischemic depolarization, min (n = 8 for each duration)		
3 min of ischemia	2.95 ± 0.78	2.77 ± 0.73
5 min of ischemia	6.62 ± 1.33	5.94 ± 0.98
7 min of ischemia	8.94 ± 2.02	9.14 ± 1.14
Neuronal damage, % (n = 8 for each duration)		
3 min of ischemia	12.2 ± 4.3	10.4 ± 3.1
5 min of ischemia	78.5 ± 23.0*	37.3 ± 12.2
7 min of ischemia	83.0 ± 20.5	86.6 ± 7.5

Values are expressed as mean ± SD.

* $P < 0.01$ compared with the nitrogen group.

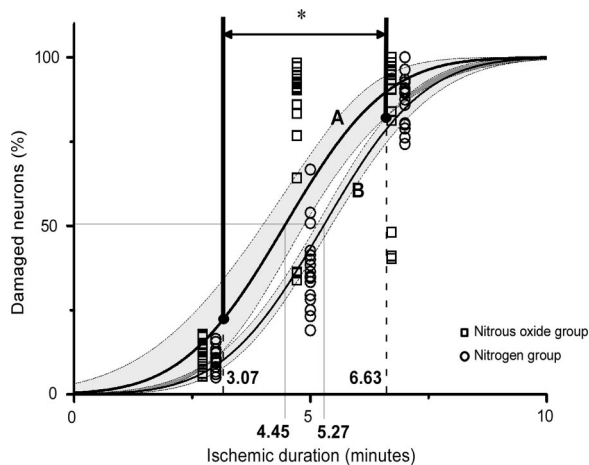


Fig. 2. Relations between ischemic duration and neuronal damage in all experimental animals. *Rectangles and circles* represent data for animals in the nitrous oxide group and nitrogen group, respectively. The logistic regression curves show close relations between ischemic duration and neuronal damage (nitrous oxide group, *line A*: $r^2 = 0.59$, $P < 0.0001$; nitrogen group, *line B*: $r^2 = 0.91$, $P < 0.0001$). The 95% confidence intervals (*shaded areas*) did not overlap from 3.07 to 6.63 min of ischemic duration (*). Ischemic durations necessary for causing 50% neuronal damage in the nitrous oxide group and nitrogen group were 4.45 and 5.27 min, respectively.

ischemia was 0.91. The percentages of neuronal damage with 3 and 7 min of ischemia were not different in the two groups.

The relations between ischemic duration and neuronal damage are shown in figure 2. The logistic regression curves indicated that the percentage of neuronal damage in the nitrous oxide group was higher in the condition of the same ischemic duration. The 95% confidence intervals did not overlap from 3.07 to 6.63 min of ischemic

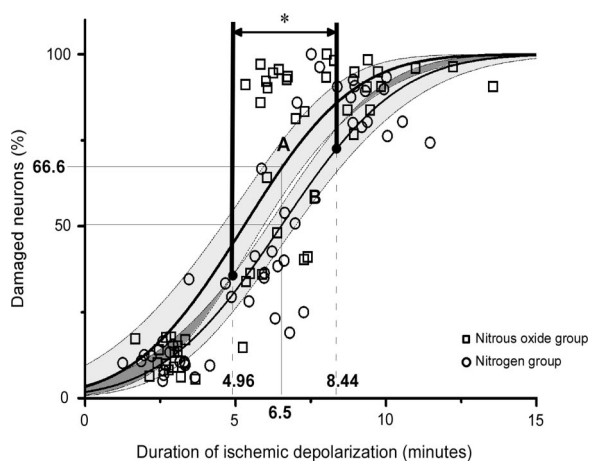


Fig. 3. Relations between duration of ischemic depolarization and neuronal damage. *Rectangles and circles* represent data for animals in the nitrous oxide group and nitrogen group, respectively. The logistic regression curves show close relations between duration of ischemic depolarization and neuronal damage (nitrous oxide group, *line A*: $r^2 = 0.64$, $P < 0.0001$; nitrogen group, *line B*: $r^2 = 0.78$, $P < 0.0001$). The 95% confidence intervals (*shaded areas*) did not overlap from 4.96 to 8.44 min of duration of ischemic depolarization (*).

duration. P_{50} -ischemia values in the nitrous oxide group and nitrogen group were 4.45 and 5.27 min, respectively.

The relations between duration of ischemic depolarization and neuronal damage are shown in figure 3. The logistic regression curves indicated that the percentage of neuronal damage in the nitrous oxide group was higher in the condition of the same duration of ischemic depolarization. The 95% confidence intervals did not overlap from 4.96 to 8.44 min of ischemic duration.

The dynamic changes in dialysate extracellular glutamate concentration are shown in figure 4. Glutamate concentrations of 5 min before ischemia (baseline values) in the nitrous oxide group and nitrogen group were 0.69 ± 0.30 and $0.69 \pm 0.22 \mu\text{M}$, respectively. In both groups, dialysate glutamate concentrations began to increase after the initiation of ischemia, increased linearly, and reached a peak at the first sample after the reperfusion (nitrous oxide group *vs.* nitrogen group: 4.15 ± 3.05 *vs.* $4.63 \pm 1.83 \mu\text{M}$). Then, dialysate glutamate concentrations rapidly decreased in the next 60 s (2.70 ± 2.59 *vs.* $2.35 \pm 1.20 \mu\text{M}$) and returned to the baseline value at 6 min after the reperfusion (0.72 ± 0.32 *vs.* $0.74 \pm 0.42 \mu\text{M}$). Mean values of maximum glutamate concentrations in the nitrous oxide group ($4.29 \pm 3.09 \mu\text{M}$) and nitrogen group ($4.63 \pm 1.83 \mu\text{M}$) were not significantly different ($P = 0.57$). Throughout the experimental period, the dynamic changes in extracellular glutamate concentration were not significantly different in the two groups ($P = 0.75$). The relative recovery rate of each dialysis probe ranged from 4.9% to 7.2%. Mean values of corrected maximum glutamate concentrations in the nitrous oxide group and nitrogen group (dialysate glutamate concentrations divided by relative recovery) were 62.9 ± 39.9 and $70.1 \pm 31.3 \mu\text{M}$, respectively. The corrected maximum extracellular glutamate concentrations were also not significantly different in the two groups ($P = 0.34$).

Discussion

In the current study, administration of nitrous oxide increased neuronal damage in the hippocampal CA1 region only with 5 min of ischemia. Logistic regression curves indicated that nitrous oxide increased neuronal damage in the condition of the same ischemic duration and shortened the ischemic duration necessary for causing 50% neuronal damage (P_{50} -ischemia) from 5.27 to 4.45 min.

As mentioned in the introduction, results of past studies on the effect of nitrous oxide on ischemic neuronal damage have not been consistent. In past studies, various methods for initiation of cerebral ischemia (anoxia,³ right carotid occlusion with hemorrhage,² and middle cerebral arterial occlusion⁴⁻⁶) were used. Furthermore, ischemia of only one intensity (ischemic duration rang-

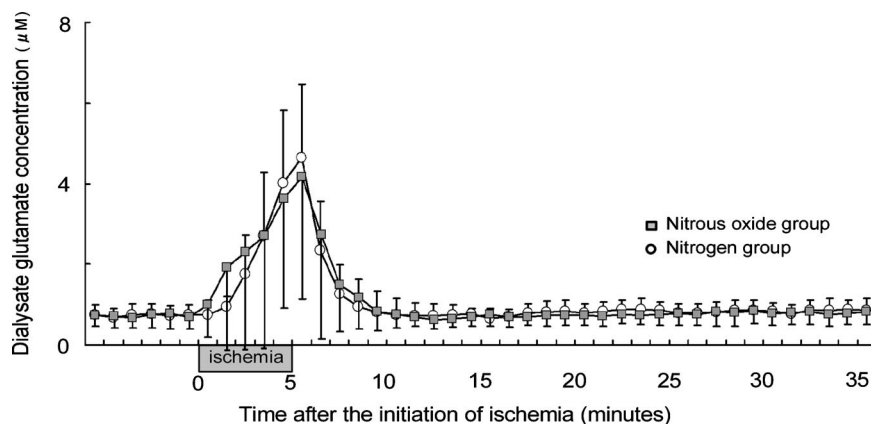


Fig. 4. Dynamic changes in dialysate extracellular glutamate concentration. Rectangles and circles represent data for animals in the nitrous oxide group and nitrogen group, respectively. In both groups, glutamate concentrations began to increase within 2 min after the initiation of ischemia. Glutamate concentration reached a maximum in the first sample after reperfusion in all animals in the nitrogen group and in five of the seven animals in the nitrous oxide group, and reached a maximum in one sample earlier or later in the other two animals in the nitrous oxide group. The mean values of glutamate concentrations in the first sample after reperfusion in the nitrous oxide group ($4.15 \pm 3.05 \mu\text{M}$) and nitrogen group (*i.e.*, maximum concentration in

the nitrogen group: $4.63 \pm 1.83 \mu\text{M}$) were significantly (6.0- to 6.7-fold) higher than those of 5 min before ischemia (nitrous oxide group: $P = 0.02$; nitrogen group: $P < 0.01$). Glutamate concentrations rapidly decrease and returned to the baseline values within 6 min. Dynamic changes in extracellular glutamate concentration were not significantly different in the two groups ($P = 0.75$).

ing from 30 to 120 min) was induced in all of those studies except for the study by Baughman *et al.*² (two intensities of ischemia). Therefore, it is difficult to evaluate the effect of nitrous oxide by measuring the change in neuronal damage with increase in ischemic intensity or by comparing results of past studies. In the current study, cerebral ischemia of three intensities was induced in the same ischemia model, and the relations between ischemic duration and neuronal damage were shown by logistic regression curves (probit curves). The use of logistic regression curves made it possible to determine the relations of ischemic duration and neuronal damage quantitatively and continuously. P_{50} -ischemia in the two groups was also determined by the use of logistic regression curves.

The gradient of logistic regression curves is steepest at the ischemic duration around P_{50} -ischemia, at which a small increase in ischemic duration causes a large increase in neuronal damage. In the current study, P_{50} -ischemia in the nitrogen group was close to 5 min, suggesting that the power of difference between groups is strongest around 5 min of ischemia. If neuronal damage had been evaluated with only 3 or 7 min of ischemia, there is a possibility that the difference in the effect of nitrous oxide during cerebral ischemia would have been overlooked.

In the current study, administration of nitrous oxide shortened P_{50} -ischemia by 0.82 min. In this context, Takeda *et al.*¹⁸ and Kobayashi *et al.*,¹⁹ in our laboratory, investigated P_{50} -ischemia values for hypothermia (34°C) and thiopental, respectively. They found that the values of P_{50} -ischemia were extended by 3.2 min (hypothermia) and 3.3 min (thiopental) compared with the value for halothane. The difference in P_{50} -ischemia caused by nitrous oxide in the current study was smaller than the differences caused by hypothermia and thiopental. Therefore, administration of nitrous oxide caused an

increase in ischemic neuronal damage, but the magnitude of its adverse effect was relatively small.

Onset time of ischemic depolarization in the nitrous oxide group was shorter than that in the nitrogen group. The difference was 0.28 min, suggesting that the cascade of neuronal damage^{7,8} began slightly earlier by nitrous oxide. One of the factors that have been shown to affect onset time of ischemic depolarization is cerebral metabolic rate,^{9,20,21} and some studies have shown that nitrous oxide increased cerebral metabolic rate.^{22,23} Although we do not have any data to show the actual cerebral metabolic rate, the results of the current study suggested that administration of nitrous oxide affects the cerebral metabolic rate.

The logistic regression curves indicated that nitrous oxide increased neuronal damage in the condition of the same duration of ischemic depolarization. For example, when the duration of ischemic depolarization was 6.50 min, the percentages of neuronal damage in the nitrous oxide group and nitrogen group were 50.0% and 66.6%, respectively. This result suggested that some factors after the onset of ischemic depolarization were involved in the increase in neuronal damage. Because extracellular glutamate release is one of major factors occurring early after the onset of ischemic depolarization and because the percentages of neuronal damage in the nitrous oxide group and nitrogen group were significantly different with 5 min of ischemia, extracellular glutamate concentration was determined with 5 min of cerebral ischemia.

To the best of our knowledge, the current study is the first study in which the effect of nitrous oxide on dynamic change in extracellular glutamate concentration was examined in such short intervals (every 60 s) during cerebral ischemia. Sampling dialysate every 60 s enabled detection of a significant difference between the two groups, which would not have been possible due to the equalization of glutamate concentration in the case of long sampling intervals. In both groups, extracellular

glutamate concentration began to increase soon after initiation of ischemia and rapidly decreased immediately after reperfusion. Because DC potentials in both groups repolarized later than 2 min after the reperfusion (*i.e.*, recovery times with 5 min of ischemia in the nitrous oxide group and nitrogen group were 2.97 ± 1.10 and 2.88 ± 0.65 min, respectively; table 3), the beginning of decrease in glutamate concentration was earlier than repolarization. This observation suggested that the decrease in glutamate concentration would be associated with washout of released glutamate by recirculation of cerebral blood flow rather than reuptake of glutamate into astrocyte by repolarization.

Despite the short sampling interval, the dynamic changes in extracellular glutamate concentration in the two groups were not significantly different. After the onset of ischemic depolarization, voltage-dependent calcium channels (mainly N- and L-type calcium channels) are activated, and calcium ions enter into the presynaptic terminal. Presynaptic glutamate is then released into the extracellular space and binds to postsynaptic ionotropic and metabotropic glutamate receptors. These receptors are activated and the activation triggers influx of calcium and sodium into the postsynaptic neuronal cells. Increase in calcium concentration in neuronal cells stimulates further glutamate release.²⁴⁻²⁷ Although nitrous oxide inhibited Ca(v)3.2 of T-type calcium currents²⁸ and ionotropic glutamate receptor-induced currents,^{29,30} nitrous oxide does not inhibit metabotropic glutamate receptors.³⁰ In addition, Amorim *et al.*³¹ reported that the values of calcium influx during hypoxia were not different with or without nitrous oxide in rat hippocampal slices. Because increase in intracellular calcium concentration correlates with glutamate release, it is likely that administration of nitrous oxide during cerebral ischemia did not change intracellular calcium concentration.

The results of the current study suggested that some factors other than extracellular glutamate concentration after the onset of ischemic depolarization were involved in the increase in neuronal damage by nitrous oxide. Hoffman *et al.*³² reported that nitrous oxide increased plasma catecholamine level and decreased neurologic score in a rat model. Pape *et al.*³³ reported that nitrous oxide (used with fentanyl) increased the value of Bax and decreased the value of Bcl-2 at 1 day and 3 days after ischemia, respectively, accelerating apoptosis in rats. Plasma catecholamine or apoptotic factor might be related to the progression of neuronal damage by nitrous oxide.

Because Kirino *et al.*³⁴ reported that the histologic change of pyramidal cells in the hippocampal CA1 region was completed within 4 days after ischemia, the percentage of neuronal damage was assessed at 5 days after ischemia in the current study. In this context, Elserly *et al.*³⁵ reported that the percentages of surviving neurons in a nitrous oxide-fentanyl group was lower

than that in the isoflurane group at 5 days after ischemia in a forebrain ischemia and hemorrhage model of rats. They also reported that the percentage of surviving neurons in an isoflurane group and nitrous oxide-fentanyl group were similar at 3 weeks and 3 months after the ischemia, suggesting that the effect of nitrous oxide on ischemic neuronal damage could have changed at more than 3 weeks after the ischemia. The long-term effect of nitrous oxide on cerebral ischemia is not clear from the results of the current study, and further investigation is required to evaluate the long-term outcome in the same model as that used in the current study.

Nitrous oxide alone has low potency, and the MAC of nitrous oxide is 104 vol% in humans³⁶ and higher (ranging from 155 to 235 vol%) in rodents.^{37,38} Therefore, in many cases, nitrous oxide is administered with other potent anesthetics such as fentanyl, barbiturates, or volatile anesthetics. In the current study, 1% halothane was administered as a basal anesthetic and 70% nitrous oxide was administered as an additional agent. Because deep anesthesia changes physiologic parameters such as blood pressure and carbon dioxide tension, depth of anesthesia may affect ischemic neuronal damage. Although anesthesia in the nitrous oxide group seems to be deeper than that in the nitrogen group, values of arterial blood gas analysis, changes in mean arterial pressure, power amplitudes of electroencephalogram, and changes in regional cerebral blood flow were not significantly different in the two groups. Therefore, the difference in anesthetic depth in the two groups did not change the physiologic parameters that might affect the severity of neuronal damage.

Values of glucose in the two groups were higher than the upper limit of the normal range. As shown in many past studies, peri-ischemic hyperglycemia worsens ischemic neuronal damage.³⁹⁻⁴¹ However, there was no difference between blood glucose values in the nitrous oxide group and nitrogen group. Therefore, in the current study, the hyperglycemic state might have caused an increase in overall neuronal damage in the two groups but would not have played a role in the increase in neuronal damage in either the nitrous oxide group or nitrogen group.

In summary, the effect of nitrous oxide on neuronal damage was quantitatively evaluated at 5 days after forebrain ischemia in gerbils. Administration of nitrous oxide increased neuronal damage only with 5 min of ischemia. The percentages of neuronal damage with 3 and 7 min of ischemia were not different with or without nitrous oxide. Logistic regression curves indicated that nitrous oxide significantly increased neuronal damage during the period from 3.07 to 6.63 min of ischemia and shortened P₅₀-ischemia by 0.82 min. Therefore, administration of nitrous oxide caused an increase in ischemic neuronal damage, but a significant adverse effect was observed with a limited range of ischemic intervals.

Furthermore, nitrous oxide did not affect dynamic change in extracellular glutamate concentration during cerebral ischemia, suggesting that unknown factors other than extracellular glutamate concentration after the onset of ischemic depolarization might be involved in the increase in neuronal damage caused by nitrous oxide.

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