INTRODUCTION

Acute use of methamphetamine (MATH) can result in toxic effects on the central nervous systems. Long term MATH use also adversely affects these systems, resulting in problems such as chronic psychosis, and paranoia (1, 2, 4, 5). The potential for serious and medically harmful effects are so significant that animal models must be used to study the more extreme adverse health effects caused by high MATH use (2, 3). Research into the influence of METH on the central nervous system (CNS) has been performed (4-6). Especially, there have been many reports describing changes in neurotransmitters, such as dopamine and serotonin (7-10). Recently, the formation of reactive oxygen species (ROS) promoting the CNS neurotoxicity induced by METH has been investigated. In particular, elevations of lipid oxidation products in the brain were reported (11-13). However, oxidative DNA damage has scarcely been reported. We have been studying about peroxidative damage in rat organs induced by METH.
In this study, in the METH-administered rat brain, we histopathologically observed peroxidatively damaged DNA, 8-OH-dG and in situ apoptosis to investigate peroxidative DNA damage.

MATERIALS AND METHODS

2.1. Animal treatment

Nine-week-old male Wistar rats weighing about 250 g were obtained from Japan SLC (Shizuoka, Japan) and maintained on a 12hr light - 12hr dark schedule with food and water available ad libitum. Methamphetamine hydrochloride (METH) (Philopon) (Dainihon-Sumitomo, Japan) was diluted in saline. The concentration of METH was adopted as the abuse dose, not a lethal dose (14).

Total 48 rats were used for this study, following protocols. All animal experiments were carried out in accordance with the principles of laboratory animal care (NIH publication NO. 85-23, revised 1985).

2.1.1. Group I: Single administration group;

Rats (n=6) were injected with 50 mg/kg (i.p.) of METH. The METH dose was near LD50. Control rats (n=6) in separate chambers were injected with saline on the same schedule as the METH group.

To observe the acute influence of METH, animals were asphyxiated with CO2 1hr after treatment.

2.1.2. Group II: Repeated administration group;

Rats (n=6) were injected with 10 mg/kg/day (i.p.) of METH for 5 days. A METH dose that would induce chronic stress was selected. Control rats (n=6) in separate chambers were injected with saline on the same schedule as the METH group.

To exclude the acute influence of METH, animals were asphyxiated with CO2 24hr after the last treatment.

2.2. Histopathological examination

For immunohistochemical analysis, METH treated rats (n=6) and control rats (n=6) were perfused through the ascending aorta with 0.01 M phosphate buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) after asphyxiation with CO2. The brain was removed and post-fixed in the same fixative overnight at 4°C. Brains were dissected out and each brain was cut into 2 mm slices. Tissue was then embedded in paraffin, and 5 μm-thick sections were cut.

2.2.1. Immunohistochemical staining for 8-OH-dG

After deparaffinization, the sections were treated with 0.3% H2O2 in methanol for 30 min for inactivation of endogenous peroxidase and then incubated in blocking solution for 10 min. For immunostaining, 8-hydroxy-2′-deoxyguanosine (8-OH-dG), RNase treatment, 250 μg/ml RNase for 1hr at 37°C, accompanied blocking. Sections were incubated with diluted anti-8-OH-dG (5 μg/ml, JICA, Japan). Immunostaining was carried out using a HISTOFINE SAB-PO(MULTI) kit (Nichirei, Japan) following the manufacturer’s instructions. Immunostaining was visualized by incubating with 0.02% 3, 3′-diaminobenzidine (DAB) and 0.03% hydrogen peroxide in PBS.

To confirm that 8-OH-dG immunopositive cells are neurons, additional immunostaining was performed with anti-human neuron specific enolase (NSE, Dako Cytomation, Japan). After 8-OH-dG immunostaining, the sections were pretreated by autoclave (121°C, 15 min) and then double stained by incubating overnight at 4°C with anti-NSE. Immunostaining was carried out using an ENVISION/ AP Kit (Dako Cytomation, Japan) following the manufacturer’s instructions. Immunostaining was visualized by incubating with Fuchsin substrate-chromogen kit (Dako Cytomation, Japan).

2.2.2. In situ apoptosis detection

In situ apoptosis detection was carried out using an ApopTag-kit (INTERGEN, USA) following the manufacturer’s instructions.

2.2.3. Semi-quantitative analysis of 8-OH-dG immunopositivity and in situ apoptosis

Neurotoxicity induced by METH was occurred in selective vulnerability region such as, nucleus accumbens. So, we observed comparatively each part of cerebrum, such as cortex, hippocampus, striatum, and nucleus accumbens, respectively.

The number of immunopositive neurons, were counted in two fields under ×100 magnification in cerebral cortex and striatum, and in all fields under ×100 magnification in the hippocampus. Also, the number of immunopositive nerve cells was counted in two fields under ×400 magnification in the nucleus accumben, respectively. Total number of positive neurons in each field was compared between the control and METH group, and the percent of control was calculated. This quantitative...
analysis was performed by someone without prior knowledge of the experimental procedure.

Statistical analysis was performed by parametric t-test, and a p-value less than 0.05 was considered significant.

All animal experiments in the present study were approved by The Committee for the Care and Use of Animals in the University of Tokushima Graduate School and performed by the Institutional Animal Care and Oversight Committee according to established guideline principles.

RESULTS

Results of semi-quantitative analysis of 8-OH-dG immunopositivity and in situ apoptosis are summarized as the percent of control in Table 1. Almost all 8-OH-dG-immunopositive cells also showed immunoreactivity of neuron specific enolase (Fig. 1). So, it was considered that peroxidative DNA damage was occurred in neurons mainly.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Percent of control for quantitative analysis of 8-OH-dG immunoreactivity and in situ apoptosis (mean ± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cerebral cortex</td>
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<tr>
<td></td>
<td>control METH</td>
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<tr>
<td>Group I</td>
<td>8-OH-dG</td>
</tr>
<tr>
<td></td>
<td>99.7 ± 5.3</td>
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<td></td>
<td>± ±</td>
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<td></td>
<td>100.0 ± 118.7</td>
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<td></td>
<td>33.3 ± 14.2</td>
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<tr>
<td>apoptosis</td>
<td>99.4 ± 9.9*</td>
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<td>± ±</td>
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<td>99.1 ± 1684.7</td>
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<td></td>
<td>38.3 ± 163.7</td>
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<td>Group II</td>
<td>8-OH-dG</td>
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<td></td>
<td>100.0 ± 106.8</td>
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<tr>
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<td>99.9 ± 163.1</td>
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<td></td>
<td>9.7 ± 21.4</td>
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<tr>
<td>apoptosis</td>
<td>120.0 ± 120.0</td>
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<td>± ±</td>
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<tr>
<td></td>
<td>8.3 ± 15.1</td>
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</table>

*; p<0.05

3.1. Group I: Single administration group

In group I, there were some individual rats demonstrating a slight spasmus as a symptom during the 1-hr experimental period (14).

Number of 8-OH-dG-immunopositive cells were 54.3±4.7 in cerebral cortex, 36.3±23.2 in hippocampus, 67.7±8.4 in striatum and 33.3±14.2 in nucleus accumbens of the control rats, respectively. Immunoreactivity of 8-OH-dG, only in the nucleus accumbens, was significantly increased, more than two times, in METH-treated rats (number of immunopositive cells ; 73.7±18.6), against the control (33.3±14.2) (p<0.05). On in situ apoptosis detection, only several apoptotic cells were observed, such as 5.7±3.8 in cerebral cortex, 4.0±1.7 in hippocampus, 11.3±1.5 in striatum and 3.7±2.9 in nucleus accumbens of the control rats, respectively. But the number of apoptotic cells was significantly increased in almost all regions, 35.3±16.2 in cerebral cortex, 57.0±12.4 in striatum and 62.3±11.1 in nucleus accumbens, respectively (p<0.05) (Fig. 2). In the hip-

![Fig. 1](image1.png)

Double staining of 8-OH-dG and human NSE in nucleus accumbens in METH-treated rat (Group II) △: neurons shows NSE-positive and 8-OH-dG negative ▲: neurons both NSE and 8-OH-dG positive

![Fig. 2](image2.png)

In situ apoptosis detection in nucleus accumbens (Group I) a: control, b: METH treated (×200)
pocampus, the number of apoptotic cells did not show the statistic significance against the control (4.0±1.7) (p= 0.18), because the individual variation was large (49.3±48.4) (Table 1).

3.2. Group II : Repeated administration group

In group II, body weight had decreased significantly by the fifth day (p<0.05) (data not shown) (14). It was suspected that systemic toxic effects of METH had appeared.

Number of 8-OH-dG-immunopositive cells were 66.3±11.2 in cerebral cortex, 73.0±7.9 in hippocampus, 33.0±3.6 in striatum and 32.7±5.5 in nucleus accumbens of the control rats, respectively. In the METH-treated rats, the nucleus accumbens only showed significant increases in 8-OH-dG-immunopositive cells (53.3±11.1) (p<0.05) (Table 1).

On in situ apoptosis detection of the control rats, relative many apoptotic cells, against the control of group I, were observed, such as 35.0±5.0 in cerebral cortex, 16.0±1.7 in hippocampus, 11.3±1.5 in striatum and 3.7±2.9 in nucleus accumbens, respectively. However, in situ apoptosis detection did not show any remarkable differences in any region in the METH-treated rats (p>0.05) (Table 1).

DISCUSSION

In METH-induced neurotoxicity, oxidative stress plays an important role. It was reported that METH administration induced elevations of lipid and protein oxidative markers in the brain (11-13). Oxidative damage differs according to the reactive oxygen species produced and the surrounding environment. In other radicals, peroxynitrite (ONOO-) participates in oxidative damage of lipids and proteins (15). In addition, the brain is a lipid-rich organ. Therefore, peroxynitrite may be the main participant in oxidative damage induced by METH in the brain. The oxidative damage in the brain might be the dominant site of lipid peroxidation. In previous our report, 4-hydroxy-2-nonenal, marker of lipid peroxidation, was enhanced in cerebral cortex and nucleus accumbens of METH-treated rat brain except hippocampus and striatum, immunohistochemically (16).

The hydroxyl-radical (·OH) is a major damage-causing free radical. While it is considered that the hydroxyl-radical participates in oxidative DNA damage, this radical has an extremely short life (17). In our other study, SOD immunoreactivity was enhanced in cerebral and cerebellar cortex, such as neurons and Purkinje cells in the METH group (16).

So, we observed the oxidative DNA damage in METH-treated rat, immunohistopathologically. In group I, immunoreactivity of 8-OH-dG was only enhanced in neurons of the nucleus accumben of METH-treated rats. On in situ apoptosis detection, positive findings were also enhanced in all examined parts compared to those in the control, though there were no significant increases in 8-OH-dG-immunopositive neurons except in the nucleus accumben. In group II, the nucleus accumben also showed enhanced 8-OH-dG immunopositivity compared to that in the control. There was no significant difference in apoptosis between the control and METH groups.

Based on our observations, it is considered that METH induces oxidative DNA damage in the brain, especially in the nucleus accumben. However, those DNA damage might be caused differently between acute and chronic administration. Then, repeated administration of METH might accentuate the active oxygen deletion system, such as SOD, which might prevent the DNA damage (13).

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REFERENCES


