

Induction of Testicular Damage by Daily Methamphetamine Administration in Rats

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Abstract

Methamphetamine (METH)-induced brain damage and apoptosis within the central nervous system are well documented. This study was conducted to investigate the toxic effects of daily METH administration on the testes in a rat model. Male Sprague-Dawley rats (5 weeks old, ~100 g, n = 64) were divided into two groups and treated with vehicle (saline, control) or METH (10 mg/kg) for 15, 30, 60 and 90 days. The results showed that daily administration of METH decreased the body, testicular and epididymis weights as well as the serum levels of total testosterone. The increased apoptotic index (*Bad/Bcl2* expression ratio) and levels of cleaved caspase-3 indicated that apoptosis had occurred in the testes of the METH-treated rats. The oxidative stress levels increased as the reduced and oxidized glutathione (GSH/GSSG) ratio decreased. The overall sperm counts decreased at 15 and 90 days, whereas morphologically abnormal sperm counts increased at 30, 60 and 90 days in the METH-treated rats. This study demonstrates that daily exposure to METH significantly reduced the number and quality of sperm in rats. The underlying pathophysiological mechanisms likely include the reduction of serum testosterone levels and the increase of oxidative stress and apoptosis in the rat testes.

Key Words: antioxidant enzymes, apoptosis, methamphetamine, oxidative stress, testosterone

Introduction

Methamphetamine (METH) is a strong central nervous system (CNS) stimulant that is abused worldwide at rates that are lower than only alcohol and marijuana (32). METH abuse has been reported to be associated with neurotoxicity. The mechanisms underlying METH-induced neurotoxicity have been identified and include oxidative stress, toxicity and apoptosis (8). Studies have demonstrated that oxidative stress is also associated with neurotoxicity induced by amphetamine, an abused substance that is structurally related to METH (41). Use of the amphetamine-derived drug 3,4-methylene-dioxymethamphetamine (MDMA; ecstasy) results in sustained hyperthermia, a slight decrease in liver weight, increased plasma

levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), and liver damage as indicated by histological analyses (17). The activities of manganese superoxide dismutase (Mn-SOD) and copper/zinc-containing (Cu/Zn) SOD significantly increased after the administration of MDMA (3, 31). SOD, catalase (CAT) and glutathione peroxidase (GPx) are naturally occurring enzymes that can scavenge reactive oxygen species (ROS). SODs, which catalyze the conversion of $\cdot\text{O}_2^-$ to hydrogen peroxide (H_2O_2), and phospholipid hydroperoxide glutathione peroxidase (PHGPx), which specifically metabolizes lipid peroxides, are both highly expressed in the testes of rats (29, 34).

Apart from the CNS, overdoses of METH have been reported to damage many organ systems includ-

ing the cardiovascular (18), pulmonary (35), renal and hepatic systems (7). The adverse effects of METH on the reproductive system have recently gained attention because METH is considered to be teratogenic and embryotoxic (42) and a study demonstrated that METH affected the abilities of male mice to mate with and to impregnate females (43). In our previous studies, the administration of amphetamine, an analogue of METH, resulted in the inhibition of testosterone production due to increased cyclic AMP production, decreased calcium channel activity, and decreased activity of enzymes such as 3β -hydroxysteroid dehydrogenase (37, 38). Moreover, the presence of METH was shown to induce apoptosis in the seminiferous tubules of mice (44). Based on the findings in the neurological systems, we hypothesized that the METH-induced oxidative stress might elicit toxic effects on the testes.

In this study, we have investigated the effects elicited by METH on the body, testicular and epididymal weights, the total testosterone levels in the serum, the METH-induced oxidative stress levels as measured by alterations in the glutathione and glutathione disulfide (GSH/GSSG) ratios, and the activities of antioxidant enzymes including SOD, CAT and GPx in the testes of a rat model. We also assessed METH-induced apoptosis in testes using an apoptotic index (*Bad/Bcl2* expression ratio) and detection of cleaved caspase-3. Moreover, we evaluated the number and quality of sperm in rats that were treated daily with METH.

Materials and Methods

Chemicals

METH was purchased from the Food and Drug Administration, Department of Health, Executive Yuan, Taiwan. All other chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, MO, USA).

Animal Treatment and Experimental Procedures

Male Sprague-Dawley rats (5 weeks old, ~100 g) were obtained from the Animal Center of National Taiwan University and were maintained on a 14-h light:10-h dark cycle and provided with food and water available *ad libitum*. The rats ($n = 64$) were randomly divided into two groups as follows: group 1 (saline-vehicle-treated control rats, $n = 32$) and group 2 (METH-injected rats, $n = 32$). Once daily, the METH-injected rats received an intraperitoneal injection of METH (10 mg/kg body weight/ml). The control rats were injected with an equal volume of saline following the same regimen as the METH-treated rats. After 15, 30, 60 and 90 days of injections, the

body weights were recorded, and blood samples were collected from the dorsal aorta using a heparinized syringe ($n = 8$, for each group and time point). The plasma samples were separated by centrifugation and stored at -80°C until all of the samples had been collected. Next, concentrations of testosterone, nitric oxide synthase-2 (NOS2) and big-endothelin-1 (big-ET1) were determined. The rats were sacrificed under deep anesthesia by isoflurane inhalation. The testes and epididymides were surgically removed and weighed. The testicular tissues harvested for RNA and protein extraction were snap-frozen in liquid nitrogen for further analysis. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Shin-Kong WSH Memorial Hospital.

Assays of Plasma Testosterone, NOS2 and Big-ET1 Concentrations

The plasma concentrations of testosterone, NOS2, and big-ET1 were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany) according to the immunoenzymatic protocols of the ELISA reader (Bio-Tek, Winooski, VT, USA). Horseradish peroxidase was used as an enzyme-labeled antigen that competed with the unlabeled antigen for binding with a limited number of antibodies on the microplates. Each concentration was calculated from a standard curve derived from five standards. The standard and sample absorbance values were monitored against a blank value at 450 nm. The data for testosterone, NOS2 and big-ET1 are expressed as nanograms (ng), units (U), and picograms (pg) per milliliter of total plasma protein, respectively.

Tissue Preparation

Testis tissues (100 g/l) were homogenized in ice-cold buffer (0.25 M sucrose, 10 mM tri(hydroxymethyl)aminomethane-HCl, and 0.25 mM phenylmethylsulfonyl fluoride, at pH 7.4) using a polytron homogenizer (IKA, Werke, Staufen, Germany). The homogenates were centrifuged at $10^4 \times g$ for 20 min at 4°C . The supernatant fractions were transferred to new tubes, and the total protein concentrations of the testis samples were measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Measurement of Total and Oxidized Glutathione in the Rat Testis Tissues

Total glutathione levels were determined according to the method previously described by Tietze (36)

with slight modifications as described (9). The total glutathione levels were assayed by the addition 10 μ l of sample to 190 μ l freshly prepared assay buffer containing 100 μ M NADPH, 5 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 1 U/ml GR, 1 mM EDTA and 50 mM phosphate buffer (pH 7.2). The change in absorbance was measured after 3 min of incubation at 450 nm using a microplate reader (Bio-Tek) and was compared to a standard curve, which ranged from 0~100 μ M. The GSG levels were determined using the method described by Griffith (11). In brief, the diluted samples or standards (70 μ l) were derivatized using 1-methyl-2-vinylpyridinium trifluoromethane sulfonate and 3.2 μ l of triethanolamine. The samples were left at room temperature for 1 h to allow the reactions to occur. The GSSG that remained was then assayed in the same manner as that for total glutathione.

Measurement of SOD, CAT, and GPx Activities

SOD activities were measured using Ransod reagents (Radox Laboratories, Antrim, UK) as described (19). In brief, the diluted standards or samples (50 μ l) were added to 1.7 ml of mixed substrate that contained 50 μ M xanthine and 25 μ M 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. Xanthine oxidase (250 μ l, 80 U/l) was then added to the mixture, and the SOD activity was measured by the degree of inhibition of this reaction at 37°C using an ELISA reader (Bio-Tek) at 450 nm for 3 min. The CAT activities in the rat testes were determined at 25°C using a Hitachi U-2000 spectrophotometer as previously described (2). Briefly, a H₂O₂ solution (59 mM H₂O₂ dissolved in 50 mM potassium phosphate buffer, pH 7.0) was added to the protein samples, and the CAT activity was measured at 240 nm for 3 min. One unit of CAT activity was defined as the degradation of 1 mmol of H₂O₂ per min. The GPx activities of the testes were determined using a Ransel kit (RS 504, Radox Laboratories) as described (14). In brief, a portion of diluted sample (20 μ l) was added to 1 ml of mixed substrate containing 4 mM glutathione, 0.5 U/l glutathione reductase (GR), 0.34 mM NADPH dissolved in 50 mM phosphate buffer (pH 7.2), and 4.3 mM EDTA. Cumene hydroperoxide (40 μ l, diluted in deionized water) was added to the mixture; the activities of GPx were measured at 37°C using a Hitachi U-2000 spectrophotometer at 340 nm for 3 min. One unit of GPx activity was defined as the amount of enzyme that catalyzed the reduction of 1 μ mol NADPH per min. The specific enzyme activities were shown as U/mg protein.

Quantification of Bad and Bcl2 mRNA Levels in Testes

The expression levels of *Bad* and *Bcl2* in the

testes of control- and METH-treated rats were measured using a quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA of each sample was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the SuperScriptIII first-strand complementary DNA synthesis system (Invitrogen) starting from 2 μ g total RNA. The qPCR was performed using a SYBR green QPCR master mix (Kapa Biosystems, Cape Town, South Africa) with gene-specific primers on the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The ratio of *Bad/Bcl2* was used as an apoptotic index for each treatment. The primers used in the qRT-PCR were the following: 5'-GGTAGGAGCTGTGGCGACT (*Bad*, forward), 5'-CAGGCCTCCTGTGGGAC (*Bad*, reverse), 5'-ATGTGTGTGGAAGAGCGTCAACC (*Bcl2*, forward), 5'-TGAGCAGAGTCTTCAGAGACAGCC (*Bcl2*, reverse), 5'-AAGGTGAAGGTCGGAGTCAA (*GAPDH*, forward), and 5'-AATGAAGGGGTCATTGATGG (*GAPDH*, reverse).

Detection of Bcl2 and Cleaved Caspase-3 Expression

The expression levels of Bcl2 and cleaved caspase-3 were determined by Western blot analysis as described (45). In brief, the protein samples from the testes of control and METH-treated rats were resolved by 14% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) followed by probing with antibodies against Bcl2 and cleaved caspase-3 (Cell Signaling, Beverly, MA, USA). The blots were also probed with an anti-GAPDH antibody (Sigma) to ensure equal protein loading. Subsequent immunoblotting procedures were performed using a chemiluminescence procedure (Millipore) as described previously (21). The changes in protein levels were assessed by densitometric scanning of the immunoreactive bands and normalization to the GAPDH loading control.

Examination of the Sperm

The caudal epididymides were dissected, weighed, placed in medium 199 (Invitrogen) and allowed to disperse. After several minutes, a small amount of sperm suspension was collected and prepared for morphological examination. The caudal epididymis was then minced in medium, incubated for 1 h at 37°C, and filtered through a nylon mesh to remove any debris. The sperm suspension was counted under a microscope using a hemocytometer to calculate the amount of sperm per weight of each caudal epididymis. The sperm suspension collected

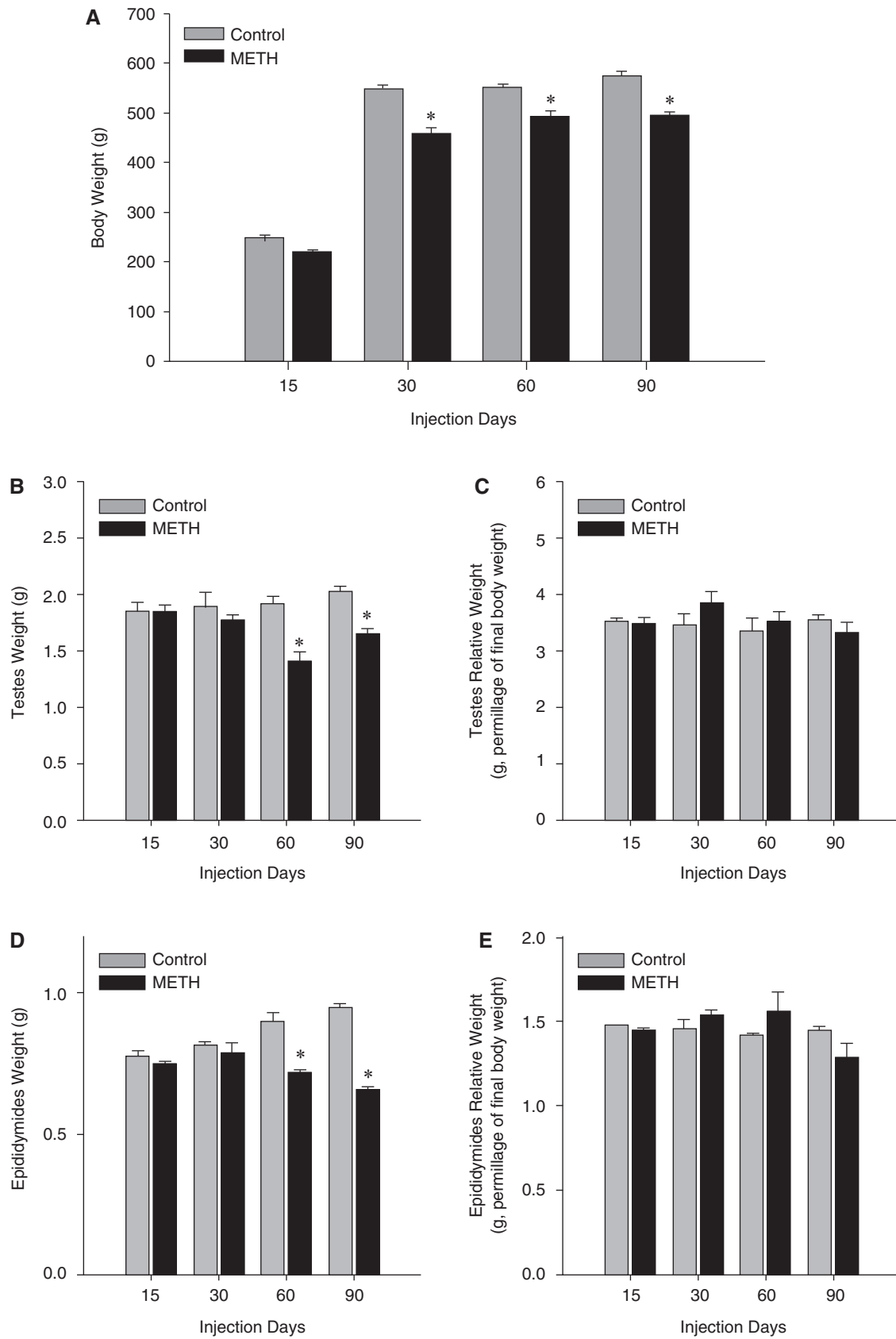


Fig. 1. Effects of methamphetamine (METH) on the body and tissue weight. (A) The body weight, (B) the absolute weight of the testis, (C) the relative weight of the testis, (D) the absolute weight of the epididymis, and (E) the relative epididymis weight of control and METH-treated rats were recorded at each time point. The data are shown as the means \pm SEM ($n = 8$). * $P < 0.05$.

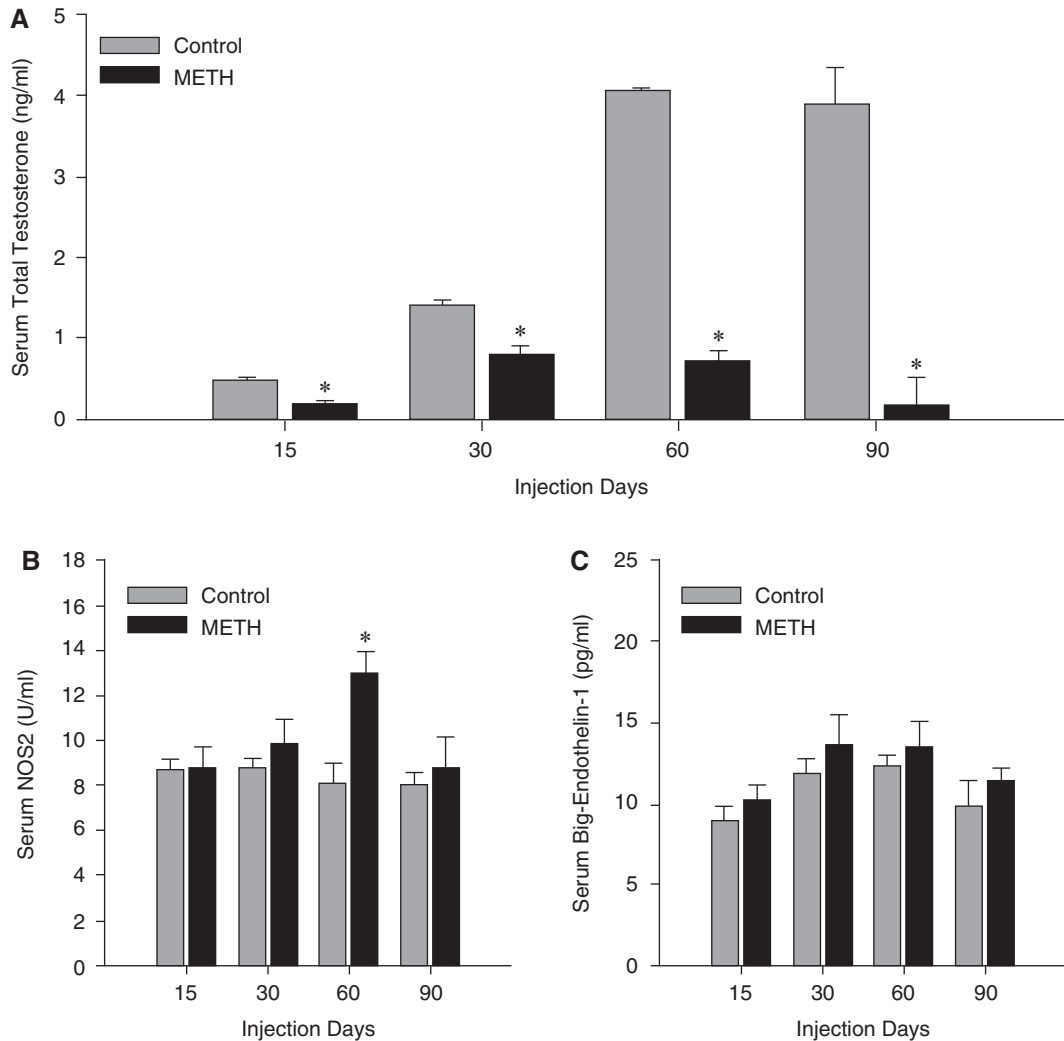


Fig. 2. Daily administration of METH decreases serum level of total testosterone. The concentrations of (A) total testosterone, (B) nitric oxide synthase (NOS)2, and (C) big-endothelin (ET)-1 in the serum were measured. Blood samples from the control and METH-treated rats were collected from the dorsal aorta, and plasma samples were separated by centrifugation. The total testosterone, NOS2, and big-ET1 concentrations were measured as described in “Materials and Methods”. The data of total testosterone, NOS2 and big-ET1 are expressed as nanograms per milliliter (ng/ml), activity units (U) per milliliter (U/ml) and picograms per milliliter (pg/ml) of total plasma, respectively. The results are shown as the means \pm SEM (n = 6). * $P < 0.05$.

for morphological examination was stained using 0.5% eosin Y, smeared onto a glass slide, air-dried overnight, fixed in methanol and examined microscopically (20). Two hundred spermatozoa in each sample were examined for head, neck and tail morphological abnormalities. Sperm without a head, with a bent tail or without a tail were considered abnormal.

Statistical Analysis

All values are presented as the means \pm SEM and then analyzed using Student’s *t*-test; *P*-values < 0.05 were considered statistically significant and marked with an asterisk.

Results

Daily Injections of METH Reduced Body and Absolute Testis/Epididymis Weights in Rats

Continuous exposure of the rats to METH significantly affected body weight gain over 15 days compared to the control rats (Fig. 1A). Treatment with METH resulted in decreased absolute testis and epididymis weights over time compared to the control rats (Fig. 1, B and D). However, despite the reduced body weight, the relative weights of the testes and the epididymides in the METH-treated rats did not statistically differ from the control rats (Fig. 1, C and E).

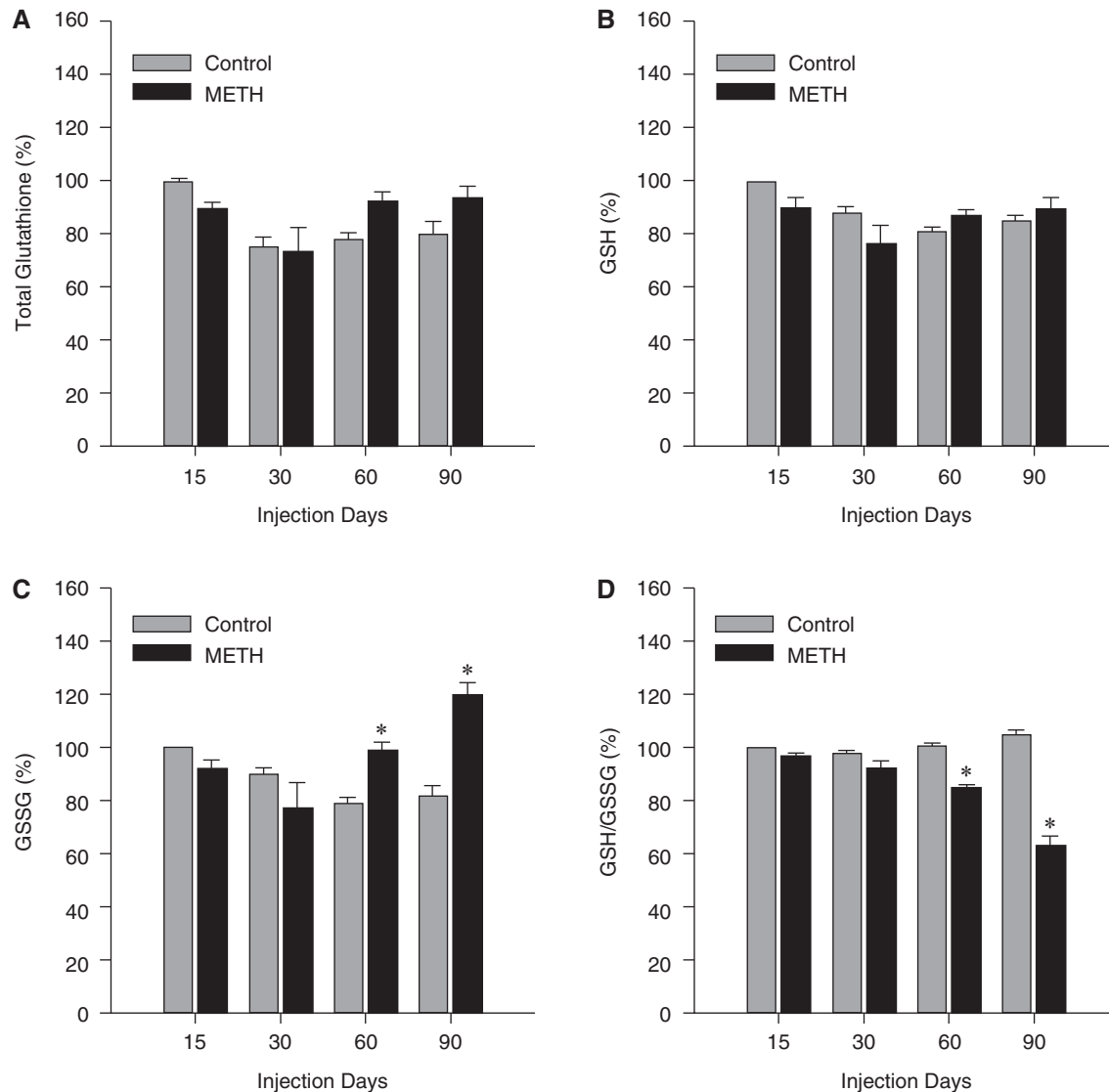


Fig. 3. Induced oxidative stress in the testis of METH-treated rats at 60 and 90 days. The levels of (A) total glutathione, (B) reduced glutathione (GSH), (C) oxidized glutathione (GSSG) and (D) the GSH/GSSG ratio in the rat testes were measured. The data are shown as the means \pm SEM (n = 6). * $P < 0.05$.

Daily Injections of METH Reduced the Total Serum Levels of Testosterone

We next determined whether the function of the testes was affected by METH treatment by measuring the total serum levels of testosterone of each rat. The administration of METH for 15, 30, 60 and 90 days significantly decreased the secretion of total testosterone compared to the control treatment (Fig. 2A). To assess the possible effects of METH on endothelial function, we further examined the serum levels of two secreted vasoactive factors, including NOS2 as a potent vasodilator and big-ET1 as a vasoconstrictor. As shown in Fig. 2, B and C, increased levels of NOS2 were only observed in METH-treated

rats at 60 days compared to the control rats (8.1 ± 0.9 vs. 13.0 ± 0.9 U/ml; $P < 0.05$), whereas the big-ET1 levels showed no difference between the METH-treated and the control rats, regardless of the time point tested.

Increased Levels of Oxidative Stress in the Testicular Tissues of METH-Treated Rats

The levels of total glutathione, GSH and GSSG, and the ratio of GSH/GSSG in the testicular tissues are shown in Fig. 3. The results showed no difference between the METH-treated and control rats regarding the levels of total glutathione (Fig. 3A) and GSH (Fig. 3B). The production of GSSG signifi-

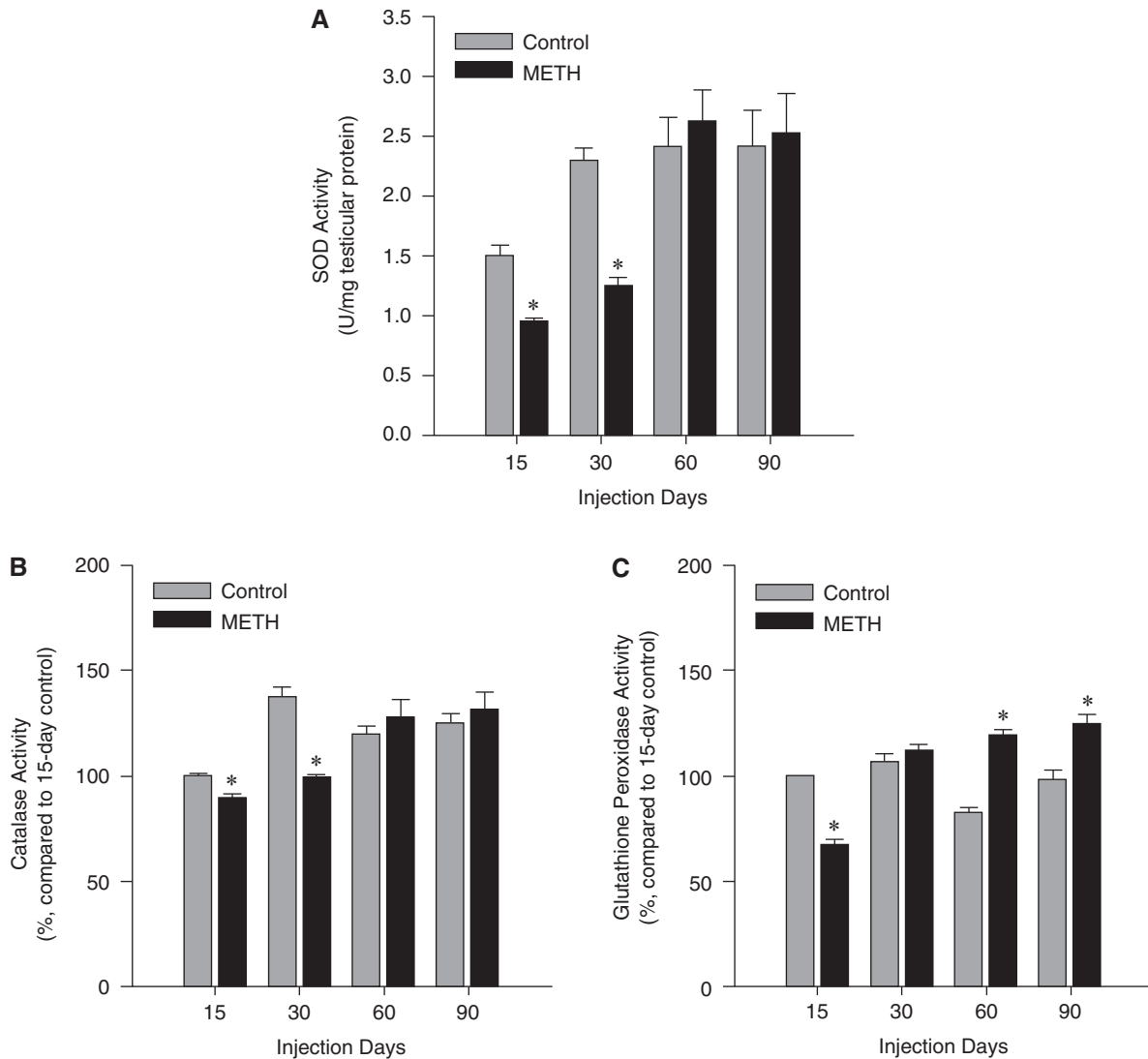


Fig. 4. Effects of daily METH exposure on antioxidant enzymes in rat testes. Compared to the control rats, the METH-treated rats exhibited significantly decreases in (A) superoxide dismutase (SOD) and (B) catalase (CAT) activities at 15 and 30 days, whereas they exhibited increased (C) glutathione peroxidase (GPx) activity at 15, 60 and 90 days in the testes. The data are shown as the means \pm SEM (n = 6). * $P < 0.05$.

cantly increased at 60 ($79.0\% \pm 2.1\%$ vs. $99.0\% \pm 2.8\%$; $P < 0.05$) and 90 days ($82\% \pm 3.8\%$ vs. $120.0\% \pm 4.2\%$; $P < 0.05$) in the METH-treated rats compared to the control rats (Fig. 3C), resulting in a significantly decreased ratio of GSH/GSSG: 60 days: $101.0\% \pm 1.3\%$ vs. $85.0\% \pm 1.8\%$; $P < 0.05$; 90 days: $104.7\% \pm 2.1\%$ vs. $63.0\% \pm 3.4\%$; $P < 0.05$ (Fig. 3). The results indicate that oxidative stress was elevated in the testes of the METH-treated rats.

Effects of METH Administration on the Antioxidant Enzyme Activities in Rat Testicular Tissues

We next investigated the activities of antioxidant enzymes including SOD, CAT and GPx in tes-

ticular tissues. The SOD activities in the testes of METH-treated rats were significantly reduced at 15 (1.5 ± 0.1 vs. 1.0 ± 0.01 U/mg; $P < 0.05$) and 30 days (2.3 ± 0.1 vs. 1.3 ± 0.1 U/mg; $P < 0.05$) compared to the control rats (Fig. 4A). However, the SOD activities exhibited no significant differences after 60 and 90 days of injections. The activities of CAT were also reduced in the METH-treated rats at 15 ($100.0 \pm 0.5\%$ vs. $90.0 \pm 1.3\%$; $P < 0.05$) and 30 days ($138.1 \pm 4.4\%$ vs. $99.0 \pm 1.4\%$; $P < 0.05$) of treatment, but exhibited no differences after 60 and 90 days of injections (Fig. 4B). The activities of GPx in the METH-treated rats at 15 days were reduced ($100.0 \pm 0.1\%$ vs. $67.8 \pm 2.3\%$; $P < 0.05$), but the activities of GPx increased after 60 ($82.9 \pm 2.3\%$ vs. $120.0 \pm 2.3\%$; $P < 0.05$)

and 90 days ($98.7 \pm 4.1\%$ vs. $125.3 \pm 4.2\%$; $P < 0.05$) of METH treatment.

Decreased Expression of *Bcl2* and Increased Levels of Cleaved Caspase-3 in METH-Treated Testes

Because the serum levels of testosterone were significantly decreased and oxidative stress was increased in the testes of METH-treated rats, we next investigated the extent of apoptosis induced in the testes by the administration of METH. The mRNA expression ratio of *Bad/Bcl2* by qRT-PCR was determined (Fig. 5A). The *Bad/Bcl2* ratio in the METH-treated testes was significantly increased at 60 (1.9 ± 0.1 vs. 3.6 ± 0.2 folds; $P < 0.05$) and 90 days (2.6 ± 0.2 vs. 3.9 ± 0.2 folds; $P < 0.05$), indicating induction of apoptosis in the METH-treated testes. When the protein expression levels of *Bcl2* and cleaved caspase-3 in the testes of METH-injected rats were examined, the results showed that the expression of *Bcl2* decreased and the levels of cleaved caspase-3 were elevated after 90 days in the METH-treated rats (Fig. 5, B and C).

Decreased Sperm Count and Increased Morphologically Abnormal Sperm in METH-Treated Rats

To determine whether the formation of sperm was compromised by the METH injection, sperm counts of the METH-injected and control rats at 15, 30, 60 and 90 days of METH treatment was performed. The sperm count significantly decreased in the METH-injected rats at 15 ($(2.1 \pm 0.1$ vs. $1.4 \pm 0.1) \times 10^6$ /g caudal epididymis; $P < 0.05$) and 90 days ($(2.7 \pm 0.1$ vs. $1.2 \pm 0.3) \times 10^6$ /g caudal epididymis; $P < 0.05$) (Fig. 6A). We further examined the abnormal sperm count in the METH-treated and control rats. Our results showed significantly increased abnormal sperm in the METH-treated rats after 15 (1.8 ± 0.2 vs. 4.2 ± 1.3 per 200 sperms), 30 (2.8 ± 0.8 vs. 8.9 ± 1.5 per 200 sperms), 60 (4.7 ± 0.2 vs. 9.3 ± 1.6 per 200 sperms) and 90 (4.3 ± 1.3 vs. 12.6 ± 2.6 per 200 sperms) days of METH treatment (Fig. 6B).

Discussion

METH alters the activity of antioxidant enzymes and increases lipid peroxidation in the mouse brain (16). A recent study has demonstrated oxidative stress responses in the adult rat retina and plasma after repeated METH treatments (22). However, our knowledge concerning METH-induced toxicity to the reproductive system remains limited (44). This study has demonstrated METH-induced toxic effects on the body, testis and epididymis weights, the release of testosterone, several markers of oxidative stress in the

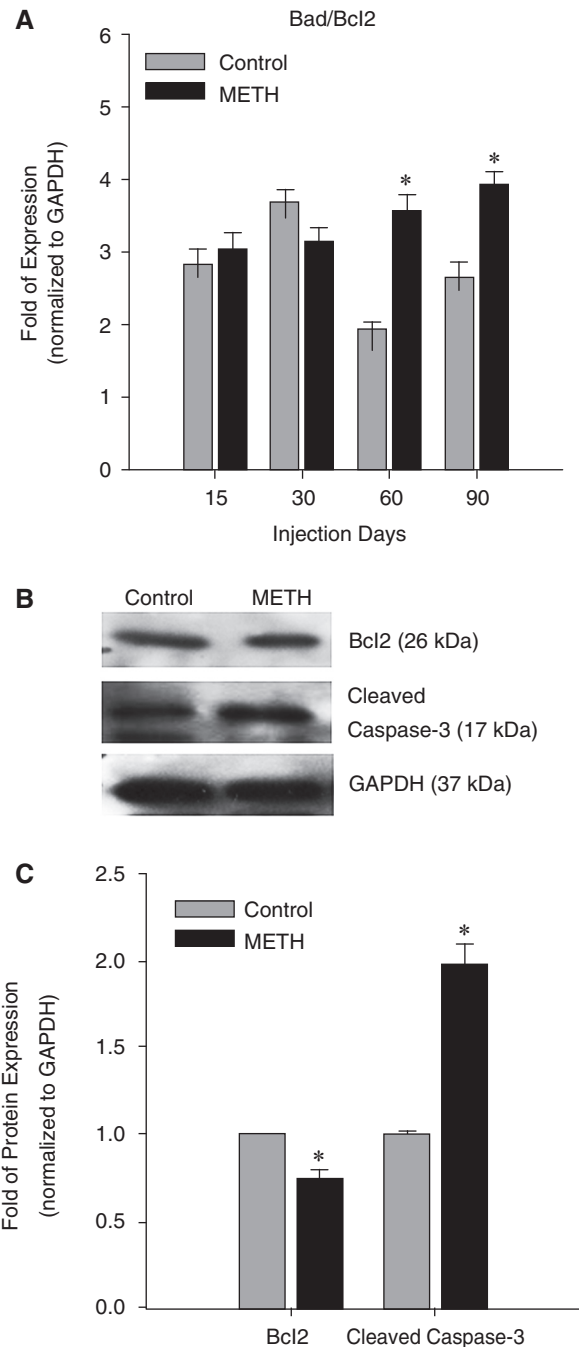


Fig. 5. Daily exposure to METH induces apoptosis in the rat testes. The mRNA ratio of *Bad/Bcl2* was assessed by qRT-PCR using *GAPDH* as the internal control (A). The mRNA ratio of *Bad/Bcl2* significantly increased at 60 and 90 days in the testes of the METH-treated rats. The data are shown as the means \pm SEM ($n = 6$). $*P < 0.05$. The protein expression levels of *Bcl2* and cleaved caspase-3 were detected by Western blot using testis samples from control and METH-treated rats after 90 days of injections. A representative result is shown in (B). *GAPDH* was used as the internal control for loading normalization. (C) *Bcl2/GAPDH* and cleaved caspase-3/*GAPDH* ratios were quantified using a densitometer. The data are shown as the means \pm SEM ($n = 4$). $*P < 0.05$.

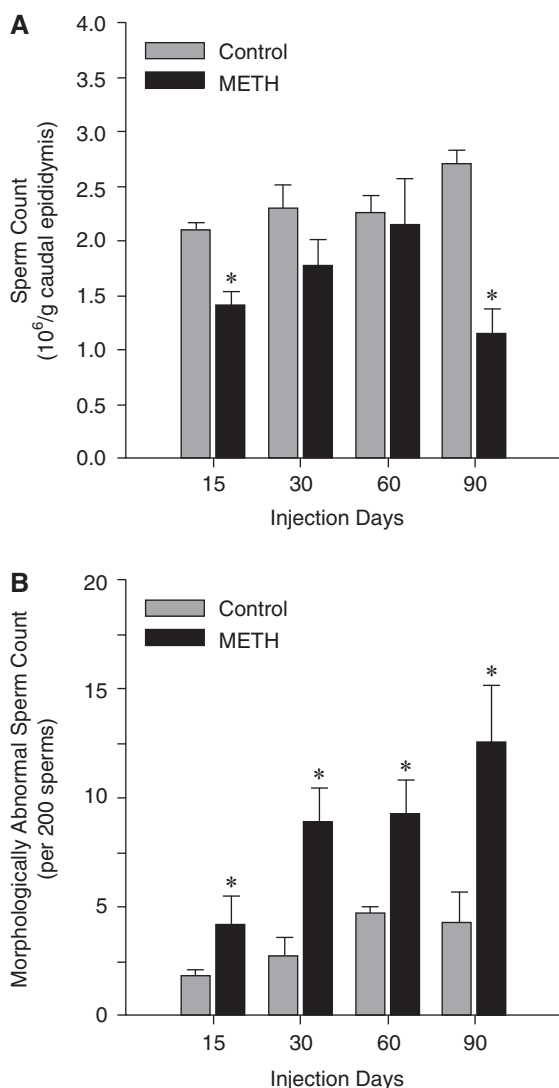


Fig. 6. Decreased sperm count and quality in the METH-treated rats. (A) Decreased sperm count was observed in the METH-treated rats at 15 and 90 days, whereas (B) increased numbers of morphologically abnormal sperm (with a lost head, bent or lost tail) were observed in the METH-treated rats at 30, 60 and 90 days. The data are shown as the means \pm SEM ($n = 6$). * $P < 0.05$.

rat testis, and on the sperm count and quality of the treated rats.

Humans who use METH for controlling food intake generally ingest doses of approximately 0.2–0.4 mg/kg, whereas those who use the drug for mood-alteration can ingest as much as 10–20 mg/kg (23). The mean maximal daily dose of Japanese METH abusers has been shown to be approximately 1.5 mg/kg (43). In this study, 10 mg/kg METH was injected once daily for 90 consecutive days. The dose was selected based on a previous study that demonstrated increased apoptosis in the seminiferous tubules of rats receiving 5 or 10 mg/kg METH for 14 days (1).

According to Roth *et al.* (33), the intraperitoneal LD₅₀ of d-METH is 15 mg/kg for mice. Another report showed that injection of 5, 10 or 15 mg/kg METH for 7 consecutive days induced apoptosis in seminiferous tubules in mouse testes (44).

Marked decreases in the body weight and the absolute weights of the testis and epididymis were observed in METH-treated rats compared to control rats (Fig. 1). The loss of body weight was consistent with previous data demonstrating the anorectic effects of this drug (4, 41). However, no differences in the food intake between METH-treated and control rats were noted. The difference in body weights between the METH-treated and control rats was beginning to be notable after 15 days of injections. The absolute weights of the testes and epididymides significantly decreased at 60 and 90 days in the METH-treated rats. Moreover, the serum testosterone levels significantly decreased in the METH-injected rats (Fig. 2A). As testosterone secretion is considered a major function of the testes, the reduced level of total serum testosterone levels in the METH-injected rats across all of the time points suggests that the testicular functions were damaged by the daily administration of METH. Cardiovascular events are among the most frequent medical complications reported in METH abusers (25). We, therefore, also assessed two endothelial markers in the serum of METH-treated and control rats. Nitric oxide (NO) serves many vital physiologic functions such as regulating vascular tone, immunomodulation, neurotransmission and penile erection (15). Endothelial NOS (eNOS), also known as NO synthase 3 (NOS3), generates NO in blood vessels and is involved in the regulation of vascular functions. Big-ET-1 is a precursor of endothelin-1 and is cleaved by endothelin-converting enzyme (ECE)-1, forming endothelin-1 (6). Our results showed that the levels of eNOS only increased at 60 days of METH treatment, whereas the levels of big-ET-1 exhibited no significant differences between the METH-treated and control rats. The chronic administration of METH in rats has been demonstrated to cause injuries to cardiac muscles (12). However, the human heart has been recently reported to exhibit lower METH uptake and retention than other tissues (40). Further investigation is warranted to clarify the effects of long-term METH treatment on cardiovascular function in rats.

The administration of METH has been demonstrated to promote self-injurious behavior in mice by activating NMDA receptors and neuronal NOS (nNOS) (26). Co-administration of the free radical inhibitors fullerene and 3-methyl-1-phenyl-2-pyrazolin-5-one-186 (MIC-186) significantly attenuated the METH-induced self-injurious behaviors (26). H₂O₂ directly and significantly inhibits the production of tes-

tosterone at least partially through a mechanism involving the reduction of cytochrome P450 side-chain cleavage (P450_{scc}) and steroidogenic acute regulatory (StAR) protein expression (39). These studies showed that the oxidative stress induced by the administration of METH is responsible for the neuronal damage observed in animal models. Studies using transgenic mice that overexpressed Cu/Zn SOD have shown that these animals were more resistant to METH-induced neurotoxicity (5). Nevertheless, there is no direct evidence delineating the mechanism by which METH treatment affects testicular oxidative stress in rats. In this study, we have shown an increase in the oxidative status and the expression of ROS-responsive enzymes in the testicular tissues of METH-injected rats. GSH is a major tissue antioxidant that provides reducing equivalents for the GPx-catalyzed reduction of lipid hydroperoxides to their corresponding alcohols and of H₂O₂ to water. In GPx-catalyzed reactions, the formation of a disulfide bond between two GSH molecules produces a GSSG molecule. The enzyme GR recycles GSSG to GSH while simultaneously oxidizing β -nicotinamide adenine dinucleotide phosphate (β -NADPH₂). When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the GSH/GSSG ratio decreases. Therefore, the ratio of GSH/GSSG and the levels of GSSG are useful indicators of oxidative stress in cells and tissues (30). Our results showed that the levels of GSSG significantly increased at 60 and 90 days of treatment and resulted in a significantly decreased ratio of GSH/GSSG, indicating elevated oxidative stress. Unlike the responses that have been reported in the brains of METH-treated rats (41), data in this study showed that the antioxidant enzyme activity in the testes decreased in response to METH treatment for 15 and 30 days. This might have been a result of METH-induced apoptosis in the testes (44), which subsequently resulted in decreased enzyme activity and decreased serum levels of testosterone. In our study, the antioxidant enzyme activities decreased in the testes of METH-treated rats but recovered at later stages to the levels observed in control rats (Fig. 4). It is possible that the increased antioxidant enzyme activities detected at 60 and 90 days were indicative of a compensatory response to counteract possible detrimental effects associated with the oxidative stress. Elevation of antioxidant enzymes in the retina, plasma and nervous system of METH-treated rats has previously been reported (24, 41). Hence, the increased activities of SOD, CAT and GPx in longer treatment in our model suggest an adaptive response to scavenge superoxide anion radicals produced during METH metabolism, which is also consistent with the observed increase in oxidative stress (GSH/GSSG ratios) at 60 and 90 days in the testes of the METH-injected rats.

Previous studies have demonstrated that reduced levels of testosterone induce apoptosis in germ cells, Leydig cells and seminiferous tubules (10, 13, 27). This study showed that the daily administration of METH reduced the serum testosterone levels and induced apoptosis as judged by the increased ratio of Bad/Bcl2 in the testes. The protein expression levels of Bcl-2 decreased and those of cleaved caspase-3 increased, in the rat testes after 90 days of METH injections. Our findings are consistent with a recent study that has described the adverse effects of METH on male reproduction (28). Apoptotic cells were detected in the seminiferous tubules of the rats that were subjected to daily injections of METH (8 mg/kg) for 14 days (28).

Decreased sperm motility was observed at 24 h in a previous study using mice that received a single 15 mg/kg dosage of METH (43). Despite the recovery of antioxidant enzyme activities at later stages of METH treatment, the sperm count was decreased and the sperm quality was affected in the METH-treated rats in the above study.

This study has presented results to show that daily exposure to METH induces apoptosis and compromises antioxidant defense in the rat testes, which results in decreased testosterone levels and a reduction in the count and the quality of the sperm in rats. Damage to the testes might also interfere with the normal functions of the reproductive system, thereby having impacts on male fertility.

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