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Regular Article

Oxidative stress status in recently abstinent methamphetamine abusers

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Aim: Methamphetamine (METH) administration is associated with excessive oxidative stress. It is not known whether the systemic oxidative stress indices would alter during early abstinence in METH abusers with positive urine testing for recent METH exposure.

Methods: Sixty-four non-treatment-seeking METH abusers enrolled from a controlled environment and 60 healthy controls participated in the study. Fasting serum malondialdehyde (MDA) levels and anti-oxidant indices, including superoxide dismutase (SOD) and catalase (CAT) activity, and glutathione (GSH) levels, were measured at baseline and 2 weeks after the first measurement. We compared the differences of these oxidative stress indices between METH abusers and controls and examined the changes of the indices 2 weeks after baseline in the METH group.

Results: At baseline, the recently abstinent METH abusers had significantly higher MDA levels, lower SOD activity, and higher CAT activity and GSH levels compared to healthy controls. CAT and GSH values were positively correlated with MDA but negatively correlated with SOD. These oxidative stress indices did not significantly correlate with age, smoking amount, Alcohol Use Disorder Identification Test scores, or METH use variables. After 2 more weeks of abstinence, the indices did not alter nor normalize.

Conclusion: Compared to controls, we found that METH abusers have persistently higher systemic oxidative stress throughout early abstinence. The compromised SOD as well as elevated CAT activity and GSH levels may act together as a compensatory mechanism to counteract excessive oxidative stress induced by METH. Whether the oxidative stress could improve after a longer period of abstinence needs to be examined in future studies.

Key words: abstinence, anti-oxidant, malondialdehyde, methamphetamine, oxidative stress.

METHAMPHETAMINE (METH) IS ONE of the most toxic drugs of abuse that leads to significant public health, legal, and environmental problems worldwide.[1] In addition to a variety of adverse mental consequences,[2–5] METH use produces serious complications systemically affecting multiple organs, including the brain, lung, heart, liver, kidney, and musculature systems.[3,6] These systemic damages are thought to result from the widespread distribution and organ toxicity of METH.[3,7] Reactive oxygen species (ROS) (such as superoxide, hydroxyl radicals, and hydrogen peroxide) and oxidative stress (which is defined as the cytotoxic consequences of ROS, including DNA damage, protein adducts, and initiate lipid peroxidation) have been shown to play an important role in METH-induced toxicity.[8–11] Malondialdehyde (MDA) is a product from lipid oxidation and has been validated as a reliable marker of oxidative stress; however, cells are equipped with

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anti-oxidant defense mechanisms, including vitamin E, glutathione (GSH) and endogenous enzymatic systems (e.g. superoxide dismutase [SOD], catalase [CAT] and glutathione peroxidase [GPX]). The imbalance of pro-oxidant and anti-oxidant in favor of the former may provoke oxidative damage.

Several lines of evidence indicate that the potent sympathomimetic actions of METH as well as the subsequent responses are the primary mechanisms underlying its toxic effects on organs.[3,12] For instance, the most extensively documented neurotoxicity is believed to depend on the METH-induced dopamine release and the ensuing increase of ROS generation and oxidative damage.[9,11,13] METH can disturb the function of the vesicular monoamine transporter, which is responsible for dopamine uptake into vesicles. After the redistribution from vesicles to cytosol, dopamine rapidly auto-oxidizes to form free radicals. Meanwhile, an excess amount of dopamine is also enzymatically converted by monoamine oxidase into dihydroxyphenylacetic acid (DOPAC) with hydrogen peroxide as a byproduct. The hydrogen peroxide can further interact with metal ions to from highly reactive hydroxyl radicals, causing further progression of oxidative damage.[11,14] Similarly, the oxidative damage caused by excessive catecholaminergic stimulation has also been shown to account for METH-induced cardiac toxicity.[10,15] Given that METH increases not only synaptic but circulating levels of catecholamine,[3,12] it presumably would cause a systemic increase of oxidative stress.

Animal studies show that METH administration can increase MDA levels,[16–19] and alter endogenous SOD, CAT, GPX activity[18–20] or GSH concentrations,[21–23] while reducing the total anti-oxidant levels.[18] The oxidative damage could be systemic, affecting multiple body organs. Pretreating rats with SOD inhibitor exacerbates the long-lasting depletions of dopamine and serotonin in the striatum caused by METH, whereas overexpression of SOD or pretreatment with anti-oxidants can protect against the striatal neurotoxicity.[24–26] In human studies, METH abusers have increased levels of MDA either in the brain[27] or blood.[28] In addition, an alteration of anti-oxidant parameters has also been observed.[29,30] These findings collectively support the association between METH use and enhanced oxidative stress.

There have been only two reports to date describing the systemic oxidative stress in METH abusers. One of them showed increased MDA levels in METH abusers[28] and the other indicated an elevation of oxidative stress by measuring various pertinent indices in the blood of amphetamine users.[30] METH is considered as a more toxic substance than amphetamine due to its cationic lipophilicity. In addition, as METH-induced oxidative stress is likely to occur during or shortly after METH exposure,[17,29] it would be of interest to understand the oxidative stress status in recently abstinent METH users. Clinical data indicate that METH withdrawal symptoms usually resolve within 2–4 weeks.[31,32] Whether oxidative stress would alter after early abstinence among METH users with a positive urine result is not yet known. In the present study, we compared the baseline levels of oxidative stress indices between METH abusers and healthy controls, and followed the changes of these indices after a further 2 weeks of abstinence.

METHODS

Subjects

All METH abusers were enrolled from the Taipei Detention Center (TDC), Taiwan, between 1 January 2007 and 30 November 2007. The TDC was established in 1952 and has become an official institution in North Taiwan responsible for preventing defendants from absconding, destroying evidence, and collusion, and consequently it allows investigations to proceed successfully. Since the Taiwan Drug Prevention and Control Act was announced in May 1998, the Rehabilitation Institution was set up in the TDC, aimed at individuals under observation and receiving treatment if necessary. In the present study, inmates at the TDC were invited to participate in the study if they fulfilled the following inclusion criteria: (i) age between 18 and 65 years; (ii) fulfilling DSM-IV diagnosis of METH use disorder; (iii) being admitted to the TDC for less than 21 days; and (iv) testing positive in urine toxicology for METH at the time they were admitted to the TDC. The urine METH test was performed by gas chromatography with the detection window of 48–72 h for recent METH exposure (manufacturer's information). The exclusion criteria were those who: (i) fulfilled the DSM-IV diagnosis of polysubstance abuse, alcohol use disorders, mood disorders, or anxiety disorders; (ii) had systemic medical illnesses, such as acute or chronic liver diseases (e.g. hepatitis B or C carrier), inflammatory rheumatologic diseases, heart failure, respiratory diseases, diabetes mellitus, or recent infections; (iii) had

major psychiatric diseases, such as schizophrenia, bipolar disorder and major depression with or without psychotic features; and (iv) had taken medications, such as psychotropics, steroids, aspirin, or other non-steroidal anti-inflammatory drugs within 4 weeks. All subjects were informed that their legal status would not be influenced by whether or not they participated in the study. After the initial assessments, eligible subjects were given a comprehensive description of the study and then recruited into the study after giving written informed consent for participation. All inmates in the TDC were allowed to smoke tobacco throughout their detention period to avoid the possible confounding effects of nicotine withdrawal.

An age- and sex-matched control group was enrolled from the Health Examination Center of Jen-Ai Branch, Taipei City Hospital, Taiwan. This group includes healthy subjects without known physical or psychiatric illnesses, which are identified by clinical interview and routine laboratory tests. They also did not meet the diagnostic criteria of drug or alcohol abuse or dependence in the past and did not drink alcohol during the previous 3 months. Blood alcohol concentration and urine drug screen both revealed negative results. The study was approved by the Institutional Review Boards of Taipei City Hospital in accordance with the Declaration of Helsinki.

Clinical diagnosis and blood samplings

METH abusers were interviewed by a psychologist (C. H. L.) according to the Chinese version of the Diagnostic Interview for Genetic Study to gather demographic information, medical history, METH use variables (including age of first METH use), a history of using METH more than 21 times in 1 year, and the occurrence of withdrawal symptoms. Clinical diagnosis of METH user disorder was further ascertained by board-certified psychiatrists (M. C. H., H. C. L.). They were also given the Chinese version of the Alcohol Use Disorder Identification Test (AUDIT)[33] for the assessment of alcohol drinking. The interval between the day of admission to the TDC (the day of urine drug screening) and the day of enrollment (the day of baseline blood sampling) was recorded as post-abstinence days. The second blood sampling was conducted 2 weeks after the first blood drawing.

Biochemical analysis

Blood samples were collected from both METH abusers and healthy control subjects via venous route. The blood specimens were centrifuged at 2000 *g* for 15 min and serum samples were stored at −80 °C until assay. The method of measuring various oxidative stress indices has been described before[34] and is briefly mentioned here. All the measurements were done in duplicates.

Determination of MDA levels

The thiobarbituric acid reactive substance assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Briefly, 100 μL of standard or each serum sample from subjects was added to appropriately labeled 5-mL vials and swirled to mix with 100 μL of sodium dodecyl sulfate solution. Four milliliters of the color reagent, which was mixed by thiobarbituric acid (TBA), TBA acetic acid and TBA sodium hydroxide, was added forcefully down the side of each vial. The vials were capped, placed in foam, added to the boiling water for 1 h, and then were immediately removed from the hot water and incubated on ice to stop the reaction for 10 min. Then the vials were centrifuged for 10 min at 1600 *g* at 4 °C, and stabilized in room temperature for 30 min. Then, 150 μL was loaded from each vial to plate and the absorbance at 540 nm was measured with a Multiskan Ex (Thermo Electron Corporation, Waltham, MA, USA).

Determination of SOD activity

Copper/Zinc-SOD levels were measured using the superoxide dismutase assay kit (Cayman Chemical Company), which utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. Then, 200 μL of diluted radical detector and 10 μL of sample were added to each. The reactions were initiated by adding 20 μL of xanthine oxidase. After being shaken carefully for a few seconds, the plates were covered and incubated for 20 min at room temperature. Absorbance values were read at 450 nm in the Multiskan Ex reader.

Determination of CAT activity

The CAT activity was measured using the catalase assay kit (Cayman Chemical Company), which

utilizes the peroxidative function of CAT and the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. In the wells containing 100 μL of assay buffer, 30 μL of methanol and 20 μL of sample, 20 μL of hydrogen peroxide was added for the initiation of the reactions. The plates were then incubated for 20 min. Thirty microliters of potassium hydroxide was added in each well to terminate the reaction, which is then followed by adding 30 μL of Purpald (chromogen) and incubation for 10 min. Ten microliters of potassium periodate was added and the absorbance at 540 nm was read using the Multiskan Ex reader.

Determination of GSH levels

Serum GSH level was measured according to the manufacturer's protocol (*Oxis*Research, Portland, OR, USA). We added serum (20 uL) and Buffer solution to each of the sample wells to a final volume of 180 uL, which was then mixed with 10-μL Solution R1 (12-mM solution of chromogenic reagent in 0.2N HCl) and 10-μL Solution R2 (30% NaOH). After 10 min of reaction in 25°C, the absorbance was read at 400 nm in an enzyme-linked immunosorbent assay reader.

Statistical analysis

Demographic and clinical data are expressed as descriptive statistics. We used the *t*-test to examine the differences of various numerical variables between the METH and control groups, and paired the *t*-test to examine the differences of numerical variables at baseline and 2 weeks after baseline in the METH group. Logarithmic transformation was used to normalize the distribution of MDA, SOD, and GSH values. Pearson's correlation was used to estimate the correlations between numerical variables. A *P*-value < 0.05 was considered statistically significant. We conducted the analyses using statistical software (PASW Statistics 18, Chicago, IL, USA).

RESULTS

A total of 64 METH subjects and 60 age- and sex-matched healthy subjects were enrolled (Table 1). For METH abusers, the age of first METH use was 25.9 ± 8.0 years and 60.9% of them had a history of using METH > 21 times in 1 year. A total of 61.3% of METH abusers reported to have withdrawal symptoms and nearly all of them had a history of tobacco use. The length of duration between the admission day to the baseline blood sampling day was 9.1 ± 4.7 days.

Expression of oxidative stress indices

Table 2 displays the oxidative stress indices in both METH and control groups. Compared to controls, the baseline serum MDA and GSH levels as well as

Table 1. Clinical characteristics of METH abusers and healthy controls

	METH group $n = 64$	Control group $n = 60$	*P*
Age, mean ± SD	31.1 ± 7.9	30.5 ± 6.9	NS
Sex			
Female	14	15	NS
Male	51	45	NS
METH use > 21 times per year, *n* (%)	39 (60.9%)	ND	–
Age of first METH use, mean ± SD	25.9 ± 8.0 (13–45)	ND	–
Duration of METH use (years), mean ± SD	5.3 ± 5.8 (0–20)	ND	–
METH withdrawal symptoms, N (%) ($n = 60$)	38 (61.3%)	ND	–
Frequency of METH use (times/month) ($n = 61$)	22.6 ± 53.3 (1–365)	ND	–
Tobacco use (pack-years) ($n = 62$)	9.0 ± 6.3 (0–30)	ND	–
AUDIT scores ($n = 63$)	4.4 ± 6.5 (0–28)	ND	–
†Interval from the day of admission to the day of baseline sampling (days)	9.1 ± 4.7 (1–21)	ND	–

†Days from the day of admission into institution to the day of baseline blood sampling.
Data are presented as mean ± SD.
AUDIT, Alcohol Use Disorder Identification Test; METH, methamphetamine; ND, no data; NS, not significant.

Table 2. Oxidative stress indices in METH abusers and healthy controls

		METH group ($n = 64$)	
	Control group ($n = 60$)	Baseline	After 2 weeks
MDA (μM)	7.8 ± 3.3	10.5 ± 5.1†	9.5 ± 4.3§
SOD (U/mL)	2.5 ± 1.4	1.7 ± 1.6†	1.8 ± 1.8§
CAT (U/mL)	61.6 ± 39.9	114.7 ± 88.0†	91.7 ± 64.2‡,§
GSH (uM)	7.3 ± 4.1	14.1 ± 9.5†	12.4 ± 8.1§

†$P < 0.05$, baseline versus control group; ‡$P < 0.05$, baseline versus 2 weeks; §$P < 0.05$, 2 weeks versus control.
Data are presented as mean ± SD. CAT, catalase; GSH, glutathione; MDA, malondialdehyde; METH, methamphetamine; SOD, superoxide dismutase.

CAT activity were significantly higher, while SOD activity was significantly lower, in METH abusers. The oxidative stress indices did not normalize even after 2 more weeks of abstinence. Compared to baseline values, the indices in METH abusers did not alter except CAT activity, which showed a reduction after 2 weeks (from 10.1 to 8.2, $P = 0.03$) but was still higher than controls. In the METH group, baseline MDA levels were positively correlated with CAT activity ($\gamma = 0.30$; $P = 0.001$) and GSH levels ($\gamma = 0.38$; $P < 0.001$), but not with SOD activity ($\gamma = 0.07$; $P = 0.44$). In addition, CAT activity and GSH levels were negatively correlated with SOD activity ($\gamma = -0.17$; $P = 0.06$ and $\gamma = -0.26$; $P = 0.04$, respectively). The baseline oxidative stress indices were not significantly correlated with age, smoking amount, AUDIT scores, and METH use patterns, such as age of first METH use, duration or frequency of METH use, or the occurrence of withdrawal symptoms.

DISCUSSION

We report here that recently abstinent METH abusers have significantly increased serum MDA and GSH levels, higher CAT activity and lower SOD activity compared to healthy controls. Importantly, the oxidative stress indices did not alter after 2 weeks. Adding to the existing knowledge that indicates METH leads to widespread organ toxicity,[3,6,7] our study further shows the systemic oxidative stress in METH abusers is enhanced throughout early abstinence.

Our finding of enhanced lipid peroxidation is in agreement with previous reports that showed human METH users have higher MDA levels in the brain[27] or in the blood.[28,30] Likewise, another study which measured thiobarbituric acid reactive substance as a proxy marker for lipid peroxidation also found the plasma levels of TBARS were significantly increased in amphetamine users. A recent animal study showed that lipid peroxidation was significantly higher in the retina, an equivalent area of central nervous system, and blood plasma of the METH-treated rats.[18] The authors also found that even after 10 days of the last dose of repeated METH treatment, the MDA level was continuously elevated.[18] Consistently, our data also demonstrated that MDA levels did not decline in METH abusers during early abstinence. These observations suggest that the toxic effect of METH may be long-term and METH abusers require a longer period of abstinence to restore from the heavy pro-oxidant stress.

SOD is the chief or first-line defense mechanism equipped in cells to neutralize ROS by converting superoxide to hydrogen peroxide, which itself can react with free ions to produce highly reactive and more toxic hydroxyl radicals (Fenton reaction). The animal studies indicate that METH can cause a decrease[20] or an increase of SOD activity[19] in the striatum, depending on the METH administration schedule and/or the interval between the administration and sacrifice of animals. To date, there have been very limited data regarding the blood SOD activity in human METH users. In the present study, a persistent reduction of SOD activity suggests a compromised elimination of superoxide radicals in our subjects. This observation is compatible with another study that showed a lower erythrocyte SOD activity in the blood of amphetamine users.[30] The

post-mortem study showed the SOD activity was instead elevated in the brains of METH abusers who tested positive for METH at autopsy.[29] The increase of SOD in the brain is considered as a compensatory response to oxidative damage produced during METH metabolism.[29] Multiple factors may contribute to the central-peripheral disparity in the results of SOD activity, such as the administered METH dose, the timing of SOD measurement, and the tissue characteristics. In Mirecki's study, the known or suspected causes of death in 17 out of their 20 subjects were related to acute METH toxicity, which was likely following the ingestion of a more toxic and higher dose than our subjects used. Meanwhile, the mean post-mortem time (15 h) at the measurement of SOD activity is much shorter than the post-abstinence time (around 9 days) at which we measured SOD in our subjects. In addition, the brain is highly vulnerable to oxidative damage due to the characteristics of high oxygen utilization, rich polyunsaturated fatty acid side chains that are prone to free radical attacks and peroxidation, and low SOD or CAT activity.[35,36] Therefore, it is highly likely that distinct adaptational responses of SOD in different tissue compartments may be triggered to counteract the METH-induced oxidative damage. Nevertheless, we suggest METH abusers have a lasting decrease of SOD activity during abstinence.

Hydrogen peroxide, a byproduct of METH metabolism, can be converted by CAT into water and oxygen in the peroxisomes or removed by conversion of reduced GSH to oxidized GSH (GSSG) to abate Fenton reaction damage. METH-associated changes in CAT activity or GSH levels are mixed across studies. For instance, METH can cause an increase[37] or decrease of CAT activity in some[20] but not all animal studies.[18] We are not aware of other clinical data examining the CAT alteration following METH use. Our results of CAT activity elevation in serum are contrary to a previous notion that amphetamine abusers have a reduced erythrocyte CAT activity.[30] The reason for this discrepant finding is not clear and might be partly due to the fact that METH is more toxic than amphetamine and presumably causes a higher production of hydrogen peroxide that requires a higher activation of CAT. Regarding GSH, the results in the literature are also inconclusive and may depend on various factors involving experimental protocols, the time at which GSH levels are measured, and the severity of oxidative damage.[38] A smaller dose of METH triggers an initial striatal GSH increase,[21] whereas high binge doses of METH could instead dose-dependently reduce the brain GSH concentration.[22,23] Likewise, it also has been noted that severe oxidative stress typically causes GSH depletion, whereas a less severe oxidative stress may result in an adaptational increase in GSH levels.[39] In human studies, Mirecki *et al*. found the post-mortem brains of their METH abusers had decreased GSH concentrations.[29] In contrast, our subjects had persistently higher CAT activity and GSH levels throughout early abstinence. Moreover, both of the two indices are positively correlated with MDA levels and negatively correlated with SOD activity. As the anti-oxidant mechanisms generally act synergistically or cooperatively in reducing oxidative damage,[40] the CAT and GSH elevation are likely to reflect a systemic demand to remove excessive hydrogen peroxide, which is a byproduct of METH metabolism, or to abate the raised levels of ROS that were displayed by higher MDA levels and weakened SOD activity as a compensatory response in the subjects.

The study has some limitations. First, the consecutive changes of oxidative stress indices over a longer period of METH abstinence, which provides a better understanding of the progression of the toxicity profile, were not examined in our study. Hence, the finding of altered status of oxidative stress is an observation limited to the recently abstinent METH abusers with a positive urine result. Second, we lacked data regarding smoking or alcohol drinking, which is known to enhance oxidative damage,[34,41] in control subjects. Moreover, only two METH subjects did not smoke, making the comparison of oxidative stress between tobacco users and non-users in the METH group difficult. Existing reports consistently show that smokers have increased lipid peroxidation as well as lower SOD activity, CAT activity and defective glutathione-related mechanisms in the blood.[42–45] While CAT and GSH elevation appears to be a distinct feature for our METH subjects, smoking may still confound the interpretation of our results and hence we cannot exclude the possibility that the case–control differences in oxidative stress indices derive from the combined effect of METH and smoking. In addition, using a cut-off score of AUDIT = 8 (greater than which is suggestive of problem drinking),[46] we found the oxidative stress indices did not differ between those METH users with an AUDIT score <8 ($n = 52$) and those with an

AUDIT score ≥8 ($n = 11$) (data not shown). Even when the cut-off score is set at 14 for the screening of alcohol use disorders,[46] all of the indices are still comparable between those with AUDIT <14 ($n = 55$) and those ≥14 ($n = 8$) (data not shown). Together with the observation that AUDIT scores were not correlated with oxidative stress levels, we speculate that the impact of alcohol drinking on our findings might be limited. Third, we did not monitor the dietary or exercise habits that may affect the profile of oxidative stress.[47] Fourth, it has been suggested that environmental stress, which is common in drug abusers and was not evaluated in our study, can also produce or augment oxidative damage.[48] Hence, we cannot infer that the heightened oxidative stress in our METH abusers is only attributed to METH per se. In line with this thinking, incarceration is likely to be stressful and could increase oxidative stress. An age- and sex-matched group of incarcerated people for crimes not involving the use of a drug that might increase oxidative stress should be a better control group for the comparison of oxidative stress indices. Consequently, future studies with simultaneous consideration of environmental factors and smoking and drinking variables might help disentangle all the potential confounding effects.

Conclusion

In summary, we observed in non-treatment-seeking METH users, that systemic oxidative stress, evidenced by increased MDA levels and compromised SOD activities, did not alter during early abstinence. In the meantime we also noticed a compensatory elevation in CAT activity and GSH levels. Whether the oxidative stress would sustain or attenuate after a longer term of abstinence needs to be confirmed in the future.

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