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The Effect of Escalating Dose, Multiple Binge Methamphetamine Regimen and Alcohol Combination on Spatial Memory and Oxidative Stress Markers in Rat Brain

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Abstract

Polydrug abuse is a major problem around the world. Methamphetamine (MA) and alcohol (AL) are two abused drugs which are frequently used together. Chronic abuse of either MA or AL causes oxidative stress in the brain and is associated with impairments in cognitive functions including various aspects of memory and learning. The present study examined the effect of escalating dose, multiple binge MA regimen, AL and MA-AL combination on spatial memory and the induction of oxidative stress in the hippocampus. Adult male Wistar rats were exposed to ethanol, an escalating dose of MA either individually or in combination for 28 consecutive days. In order to examine the short- and long-term effects of chronic exposure to the drugs, each group was then subdivided into two further groups. Thereafter, spatial memory was tested using a Morris water maze, either one day or 14 days after the drugs were withdrawn. At the end of the behavioral testing, oxidative stress markers including Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), Catalase (CAT), and Malondialdehyde (MDA) were measured. Our results showed that MA, but not AL, impaired spatial memory. Although AL alone had no effect, it exacerbated the impairment due to MA when the drugs were co-administered. In addition, while both drugs significantly induced oxidative stress in the hippocampus when given alone, co-administration of these drugs resulted in a greater oxidative stress and an impairment of the antioxidant enzyme glutathione peroxidase in rat hippocampus. Taken together, this study demonstrates that MA in combination with AL has synergistic effects on increased oxidative stress in the hippocampus, as well as spatial memory impairment.

Keywords: Methamphetamine; Alcohol; Spatial memory; Oxidative stress; Hippocampus

Introduction

Prevalence of polydrug abuse is increasing and becoming more widespread in recent decades [1-3]. This is of concern because of the adverse health and social consequences of polydrug abuse [4]. Alcohol (AL) and Methamphetamine (MA) are two of the most widely consumed drugs, which are frequently used together [5-8]. MA is an illicit amphetamine derivative, with a high potential for abuse and dependence [9]. It is one of the most popular psychostimulants among young people which is used as a recreational drug (party drug), because of its ability to enhance mood, energy, exhilaration and euphoria [3]. In addition, the effect of MA in suppressing appetite makes it attractive to young women [10,11]. Unlike the acute effects of a single low dose of MA, which can improve cognitive processing speed, attention, concentration, and psychomotor performance [12], chronic MA abuse results in profound neurotoxicity and cognitive deficits [13-15], which may persist for months and even years after withdrawal [16,17]. In fact, MA cause neurodegeneration in various brain regions which are involved in cognitive processes, such as hippocampus, a structure located in the medial temporal region of the brain [18,19]. The hippocampus is critical for learning and memory formation, particularly for spatial memory and navigation [20-22]. Although, chronic MA exposure primarily results in damage to dopaminergic neurons in the striatum, there is evidence that MA is also neurotoxic to both serotonin and norepinephrine nerve terminals in the hippocampus [23]. Furthermore, MA can produce neuronal damage in the hippocampus. There is increasing evidence that chronic MA abuse causes degeneration of pyramidal neurons in the CA1 and CA3 regions of the hippocampus and granular cells in the dentate gyrus [24,25]. The MA- induced neuronal degeneration results in reduced hippocampal

volume and, ultimately, impairment of hippocampal-dependent cognitive function [26,27], which is reflected in behavioral deficits in hippocampus- dependent memory tasks including Morris Water Maze (MWM) [28,29].

Although the precise mechanisms underlying MA-induced hippocampal neurodegeneration are still unclear, there is evidence that oxidative stress is involved in MA neurotoxicity [30,31]. Recent studies have demonstrated that MA has induced mitochondrial damage via production of ROS in rat hippocampal neural progenitor cells [30,31]. However, MA toxic effects depend largely on the pattern of abuse [32,33]. Most of the studies underlying MA-induced neurotoxicity have used the single day-single dose or single day-multiple dose administrations [34-36], which are not thought to simulate the human MA abuse pattern. This pattern often begins with a period of relatively gradual dose escalation [5,37]. It has been suggested that an escalating dose binge pattern of MA exposure may afford partial protection against its deleterious effects that occur most frequently during high-dose MA abuse [38,39].

AL is a general Central Nervous System (CNS) depressant and also one of the most widely abused drugs in many societies [40-43]. Long-term heavy AL consumption may damage the brain's structures and functions [44-46], particularly hippocampal damage [47,48]. There is growing evidence that oxidative stress plays an essential role in alcohol-induced hippocampal damage [49,50]. The prevalence of MA and AL co-abuse is of concern because this drug combination can have serious and unpredictable consequences than either drug taken alone at the same dose [8,51,52].

To our knowledge, there are no previous studies investigating the effect of escalating dose binge pattern of MA and AL co-abuse on spatial memory and the induction of oxidative stress in the hippocampus. In the present study, we treated adult male rats with AL and/or an escalating dose-multiple binge MA exposure. Then spatial learning and memory as well as oxidative stress parameters markers in the hippocampus were measured.

Materials and Methods

Subjects

Male Wistar rats (220-240 g) were used in this study and housed in pairs at the temperature of 21.0 ± 2°C and on a 12 h reverse light/dark cycle (lights on 7:00 a.m.). Food and water were provided ad libitum, and body weights were recorded daily throughout the treatment and withdrawal periods. All procedures were approved by regional ethics committee of Tabriz University of Medical Sciences.

In the beginning, 64 rats were randomly divided into four experimental groups: sham, Methamphetamine-treated (MA), Alcohol-treated (AL) and methamphetamine and alcohol-treated (MA+AL) groups, with 16 rats in each. After the cessation of drugs, each group was divided into two subgroups, with 8 rats in each. One group was tested by Morris water maze one day and other was tested 14 days day after the drugs withdrawal. So, there were 8 groups as follow: Sham (1-day wd), AL (1-day wd), MA (1-day wd), AL+MA (1-day wd), Sham (14-day wd), AL (14-day wd), MA (14-day wd), AL+MA (14-day wd).

Drugs

D-Methamphetamine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in saline and administered subcutaneously (s.c.) doses represented free base. Alcohol (absolute) was purchased from Merck Co. (Germany) and administered intragastrically by gavage, 10 min before administration of saline and MA.

Drugs Administration

All animals were administered either saline or alcohol (2 g/kg) via gavage once a day for 28 consecutive days. They were also injected with either MA or saline. All rats were initially exposed to the ED-phase of drug administration (starting with 0.1 mg/kg and escalating to 4.0 mg/kg). During this phase, the animals received three daily subcutaneous injections of either MA or an equivalent volume of saline for 14 successive days, as described in Table 1. On the day following the ED-pretreatment, the animals were exposed daily for 14 successive days to a high-dose MA binge, each binge consisting of four successive injections of 6.0 mg/kg MA at 2h intervals [24].

Blood AL concentration

Blood Alcohol Concentration (BACs) was determined as previously described [52]. Blood samples were obtained from AL and MA + AL groups, 2 h after the last alcohol dose (Table 1).

Morris Water Maze

Either 1 day or 14 days after the cessation of drugs, spatial learning and memory was assessed using a Morris Water Maze (MWM) task. A black circular water pool (with a diameter of 200 cm and a depth of 60 cm) was filled with water at 23±1°C temperature and placed in a room with many visual cues on the walls. The behavior of rats was tracked with a camera that was placed directly above the pool and connected to a computer.

Hidden platform task

Animals were trained on the hidden platform task to assess spatial acquisition. A black escape platform (10 cm diameter) was submerged 2 cm below the water surface in one quadrant region in the pool and remained in the same location for all trials. The animals received three blocks of training, each consisting of four trials (12 trials in total). Each block was considered as a separate test session and the blocks of trials were separated by 30 min. All training was completed in a single day and took place during the light cycle. In each trial, the rats were permitted to swim up to 60 s to find the escape platform. Once on the platform, the rats remained there for 15 s before starting the next trial. After completion of the fourth trial of the block, rats were removed from the pool and placed in a temporary holding cage under a heat lamp. Escape latency (the time to find the platform), path length (the distance travelled to reach the platform), and swim speed were recorded and analyzed.

Probe trial

Spatial memory retention was evaluated in a probe trial that carried out 24 h after the last acquisition trial. The platform was removed and the animal was left to swim in the pool for 60 seconds. The percentage of time spent and distance traveled in each quadrant was recorded.

Visible platform task

Animals were submitted to a visible platform task to test eyesight and swimming ability. The rats were trained to escape to a visible platform, which was moved to a different maze quadrant on each trial. Each rat was given four trials in which they were allowed to search the platform for a period of 60 sec.

Oxidative Stress Parameters

Tissue Preparation: The rats were sacrificed by cervical dislocation under ether anesthesia 24 h, after the probe trial. The hippocampus was rapidly dissected on an ice-cold surface, and frozen in liquid nitrogen and subsequently stored at−80°C until biochemical analysis. The frozen hippocampal tissues were homogenized in cold phosphate buffer (0.1 M, pH 7.4), containing 140 mM KCl and 1 mM EDTA [53]. The homogenates were centrifuged at 1000 rpm for 10 min at 4°C, and the supernatant was used in the experiment.

Superoxide Dismutase Activity: Superoxide Dismutase (SOD) activity was determined using a RANSOD kit (Randox Labs, Crumlin UK) according to the method of Delmas-Beauvieux, et al. [54]. In this method, xanthine and xanthine oxidase were employed to generate superoxide radicals that react with 2-(4-iodophenyl)-3 (4-nitrophenol)-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. The SOD activity was measured the degree of inhibition of this reaction at 505 nm using a spectrophotometer. The activities were expressed as units per milligram of protein (U/mg protein).

Glutathione Peroxidase Activity: Glutathione Peroxidase

(GPx) activity was determined according to the method of Paglia and Valentine [55] using a RANSEL kit (Randox labs. Crumlin UK Randox). GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and Nicotinamide Adenine Dinucleotide Phosphate (NADPH), the oxidized glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance was measured spectrophotometrically at 340 nm. The activities were reported as U/mg protein.

Catalase Activity: CAT activity was determined spectrophotometrically according to the method of Aebi [56]. The assay was based on measuring the decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm. The activities were expressed as U/mg protein.

Malondialdehyde: Malondialdehyde (MDA) concentration as an indicator of lipid peroxidation was determined according to the method of Draper and Hadley [57]. In this method, the content of malondialdehyde (MDA) as Thiobarbituric Acid Reactive Substances (TBARS) was assayed using a spectrophotometer at 532 nm. The results were expressed as nmol/mg protein.

Statistical analysis

Data from the acquisition phase of the MWM task were analyzed by repeated-measures three-way ANOVA with MA treatment (MA vs. Saline), AL treatment (AL vs. saline) and withdrawal period (1 d withdrawal vs. 14 d withdrawal period) as between subject factors, and training blocks as within subject factor. Data from the visible platform task and the probe trial, as well as all biochemical measures were analyzed using three-way ANOVA (MA treatment × AL treatment × withdrawal period). Statistical analysis of body weight changes was conducted using a repeated-measures two-way ANOVA with MA treatment and AL treatment as between group factors and week as within group factor.

All analyses of variances were followed by the Tukey's test for multiple comparisons, whenever appropriate. Differences with $p < 0.05$ were considered statistically significant. Data were expressed as mean ± SEM.

Results

Mortality

There was no mortality in the Sham or AL groups. However, one animal in the MA group and two animals in the MA+AL group died before the end of the study.

Plasma Al Concentration

The results showed that BAC level was increased in both AL and MA+AL groups (AL group: 0.92 ± 0.10 mg/ml; MA+AL group: 0.85 ± 0.13 mg/ml). Although, the BAC level was higher in AL rats compared to MA+AL rats, but the difference was not statistically significant (data not shown).

Weight Gain Data

Statistical analysis of body weight changes showed significant effects of week (F(4,54)=6.838, p<0.0001), MA treatment (F(1,57) =565.478, p < 0.0001), as well as week × MA treatment interaction (F(4,54) =153.201, p < 0.0001) on weight gain. No significant main effect AL treatment, AL × MA and week × AL interactions on weight gain was detected (data not shown). The weekly body weight average is

depicted in Figure 1. As shown in this figure, there was no significant difference in body weight between groups before exposure to the drugs (pre). During the period of drugs administration, MA-treated rats significantly lost weight compared with rats that were not treated with MA (weeks 1-4). The body weight loss continued even after MA was withdrawn (weeks 5-6) (Figure 1).

Behavioral Results

Data from the Morris water maze task are shown in Table 2. Repeated-measures three-way ANOVAs revealed a significant main effect of block on time latency (F(2,52)=159.312, p<0.0001) and path length (F(2,52)=230.468, p<0.0001) to find the hidden platform. The effect of MA treatment, and its interaction with block were also significant for both escape latency (F(1,53)=123.641, p<0.0001, and F(2,52)=11.750, p p<0.0001, respectively) and path length (F(1,53)=138.669, p<0.0001, and F(2,52)=5.458, p<0.001, respectively). As seen in Figure 2A and 2B, there were significant differences in path length and latency to find the hidden platform between the MA-treated and the saline-treated rats. Indeed, the MA-treated rats showed less improvement across the training trials than the rats that were not treated with MA. Similarly, the statistical analysis displayed significant MA × withdrawal period interaction on escape latency (F(1,53)=11.290, p=0.001) and path length (F(1,53)=6.269, p<0.05). Figure 3 illustrates the effect of drug withdrawal period on the performance of MA- and saline-treated rats
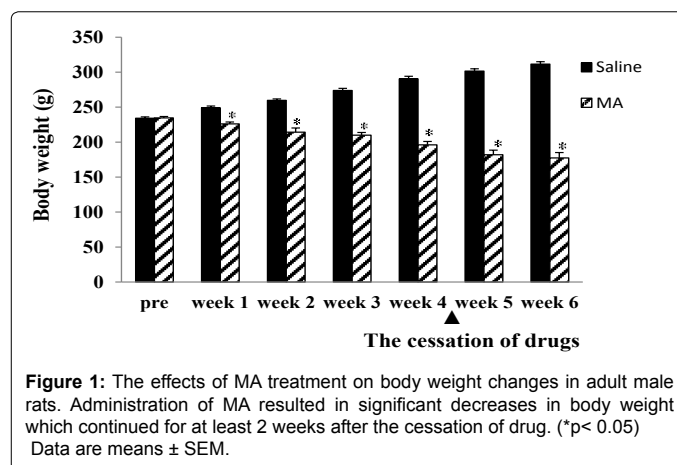
Figure 1: The effects of MA treatment on body weight changes in adult male rats. Administration of MA resulted in significant decreases in body weight which continued for at least 2 weeks after the cessation of drug. (*p< 0.05) Data are means ± SEM.

Day	Time (MA dose mg/kg)		
	09:00	12:15	16:30
1	0.1	0.2	0.3
2	0.4	0.5	0.6
3	0.7	0.8	0.9
4	1.0	1.1	1.2
5	1.3	1.4	1.5
6	1.6	1.7	1.8
7	1.9	2.0	2.1
8	2.2	2.3	2.4
9	2.5	2.6	2.7
10	2.8	2.9	3.0
11	3.1	3.2	3.3
12	3.4	3.5	3.6
13	3.7	3.8	3.9
14	4.0	4.0	4.0
15-28	(4×6.0 mg/kg; 2 h intervals)		

Table 1: Escalating dose schedule of MA administration in adult male rats.

Groups	Hidden platform task						Probe task (the target quadrant)	
	escape latency (sec)			path length (cm)			time spent (%)	travelled distance (%)
	Block 1	Block 2	Block 3	Block 1	Block 2	Block 3		
Sham (1-day withdrawal)	28.41 ± 4.5	17.00 ± 2.0	9.66 ± 2.5	523.74 ± 125.8	317.14 ± 59.4	126.67 ± 48.4	29.57 ± 5.7	29.93 ± 5.1
AL (1-day withdrawal)	26.13 ± 7.1	17.87 ± 4.4	8.74 ± 3.1	531.51 ± 120.9	312.06 ± 78.1	127.72 ± 31.6	34.81 ± 5.2	28.31 ± 3.9
MA (1-day withdrawal)	27.35 ± 4.4	19.25 ± 2.4	12.66 ± 2.7	530.15 ± 154.8	301.07 ± 74.0	193.35 ± 49.4	26.09 ± 6.9	26.40 ± 5.8
AL+MA (1-day withdrawal)	26.19 ± 4.6	16.13 ± 4.2	10.45 ± 2.4	530.12 ± 130.4	311.25 ± 97.2	129.68 ± 42.8	27.30 ± 5.8	26.99 ± 6.1
Sham (14-day withdrawal)	30.44 ± 4.2	23.48 ± 4.1	19.64 ± 3.4	717.83 ± 185.9	511.63 ± 121.2	245.92 ± 79.3	20.41 ± 4.5	19.36 ± 4.9
AL (14-day withdrawal)	30.41 ± 6.3	31.76 ± 4.6	23.64 ± 4.2	750.32 ± 102.3	602.46 ± 159.7	289.35 ± 86.9	18.28 ± 3.9	19.67 ± 3.9
MA (14-day withdrawal)	31.09 ± 5.9	25.01 ± 3.2	20.55 ± 4.4	684.44 ± 154.3	473.55 ± 97.2	187.85 ± 62.9	18.64 ± 6.0	21.12 ± 5.6
AL+MA (14-day withdrawal)	34.32 ± 6.1	27.18 ± 5.2	25.17 ± 5.5	752.93 ± 172.3	522.54 ± 116.7	301.65 ± 70.1	22.90 ± 3.6	20.16 ± 3.8

Table 2: Mean values for various measures in the Morris water maze (Data are shown as means ± SEM).

Figure 2: The effect of MA treatment on A) escape latency and B) swim path length to find the hidden platform and C) swim speed during the three acquisition blocks. MA significantly impaired spatial learning performance in the MWM, as evidenced by significantly longer escape latency and swim path length in MA-treated rats compared to saline-treated rats (*p<0.05). MA administration increased the speed of swimming in MA-treated rats compared to saline-treated rats (*p<0.05). Data are means ± SEM.

Figure 3: The effect of withdrawal period on acquisition of spatial memory in MA-treated rats. Panel A shows the effect of withdrawal period on escape latency to find the hidden platform. Panel B shows the effect of withdrawal period on swim path length to find the hidden platform. There were significant differences in path length and latency to find the hidden platform between the MA-treated and the saline-treated rats (*p<0.05). However, in rats withdrawn from MA for 14 days, this difference was significantly greater than rats withdrawn from MA for 1 day. Data are means ± SEM.

in the acquisition task. MA-treated rats withdrawn from MA for either 1 or 14 days required more time and swam greater distances to reach the hidden platform, compared with saline-treated rats. However, in rats withdrawn for 14 days, these differences were significantly greater than rats withdrawn from drug for 1 day. Furthermore, neither the main effect of AL nor withdrawal period was significant for these parameters (data not shown). Finally, no other statistically significant two- or three-way interactions were detected (data not shown) (Figures 2 and 3).
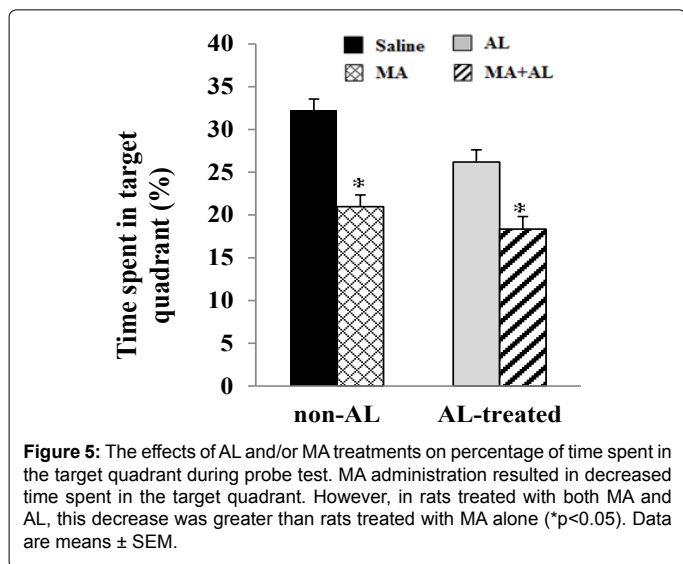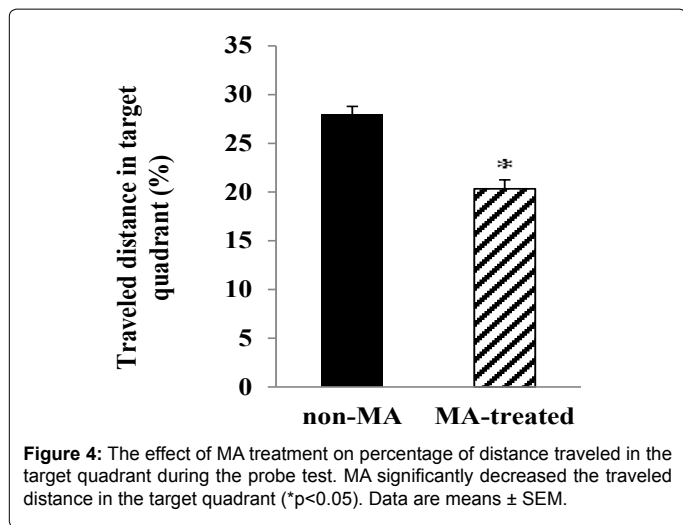
A repeated three-way ANOVA on average swim speed during the acquisition task showed a significant main effect only for MA treatment (F(2,144)=4.862, p<0.05). Furthermore, Figure 2C shows that MA-treated rats swam faster than the rats that were not treated with MA. No other main effects or interactions were significant (data not shown).
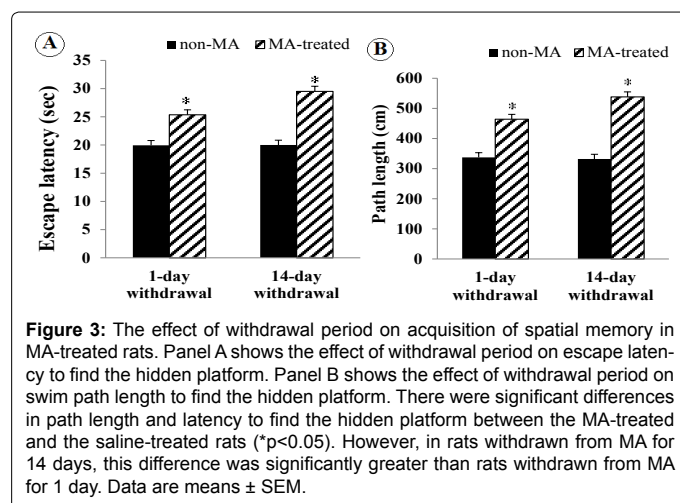
A three-way ANOVA revealed a significant effect of MA treatment on the time spent (F(1,53)=41.939, p<0.0001) and traveled distance (F(1,53)=35.580, p<0.0001; Figure 4) in the target quadrant. The MA × AL (F(1,53)=5.513, p<0.05; Figure 5) interaction was also significant for the time spent in the target quadrant. As illustrated in Figure 6, rats treated with both MA and AL exhibited more impaired performance on the probe trial than rats treated with either MA or AL alone, as well as saline-treated rats. Moreover, no significant main effects of AL treatment and withdrawal period, as well as their interaction on either the time spent or the traveled distance in the target quadrant were observed (data not shown). Finally, the three-way ANOVA failed to show significant effect of MA × AL × withdrawal period interaction on these parameters (data not shown) (Figures 4 and 5).

Oxidative Stress Measurements

Three-way ANOVA revealed only a significant main effect of MA treatment (F(1,53)=14.789, p<0.0001) on SOD activity, indicating that the level of SOD activity was decreased significantly following MA

Figure 4: The effect of MA treatment on percentage of distance traveled in the target quadrant during the probe test. MA significantly decreased the traveled distance in the target quadrant (*p<0.05). Data are means ± SEM.

Figure 5: The effects of AL and/or MA treatments on percentage of time spent in the target quadrant during probe test. MA administration resulted in decreased time spent in the target quadrant. However, in rats treated with both MA and AL, this decrease was greater than rats treated with MA alone (*p<0.05). Data are means ± SEM.

administration (Figure 6A). Neither the main effect of AL treatment, nor the main effect of withdrawal period was found to be statistically significant. Similarly, none of the two- or three-way interaction effects were statistically significant (Figure 6).

A three-way ANOVA conducted on GPX activity failed to show significant effects of AL treatment, MA treatment and withdrawal period (data not shown). In addition, except for the AL×MA interaction (F (6,138)=8.148, p<0.01), the analysis revealed no significant two- or three-way interaction(data not shown). In fact, as shown in Figure 6B, the GPX activity was significantly lower in MA+AL group than control group.
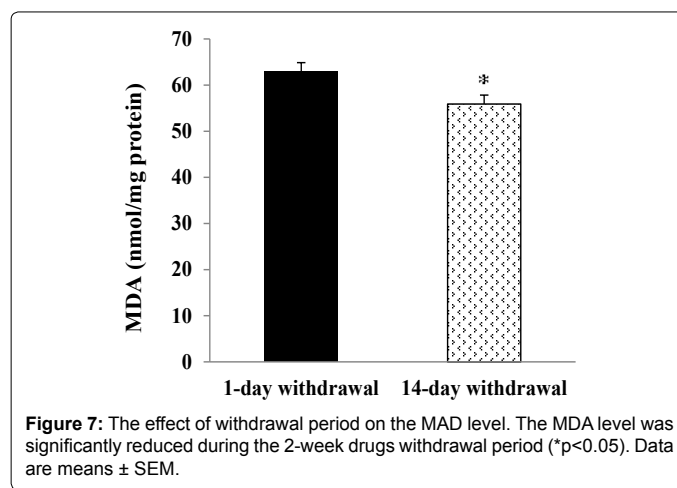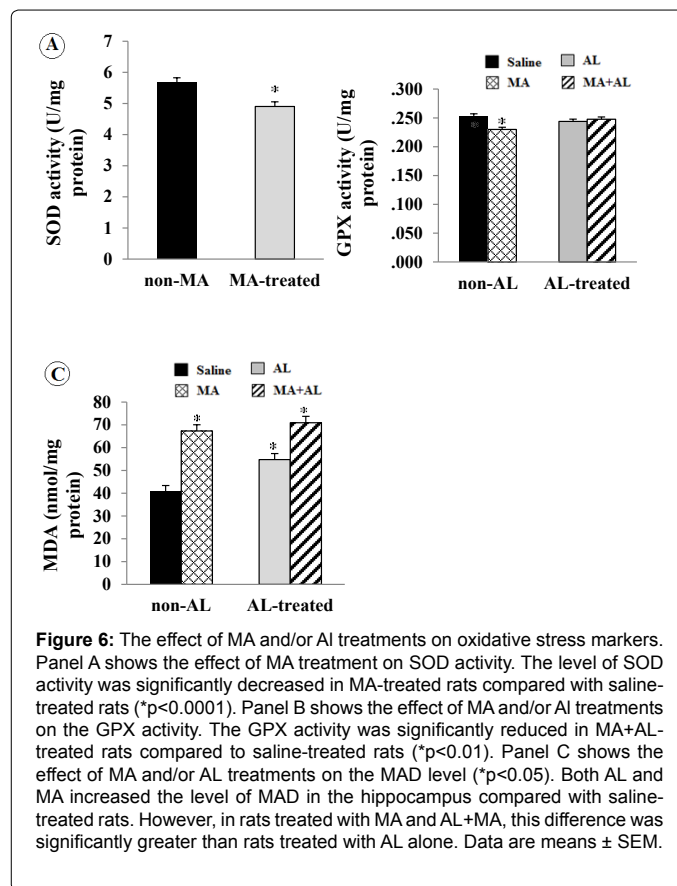
Statistical analysis of MAD level with three-way ANOVA revealed significant main effects of AL (F(1,53)=6.308, p<0.05), and MA (F(1,53)=74.203, p<0.0001) treatments, as well as withdrawal period (F(1,53)=6.813, p<0.05), as well as AL×MA interaction (F(1,53)=7.021, p<0.05). As can be seen from Figure 6C, the MDA level was significantly higher in the rats exposed to either AL or MA compared to saline-treated rats. Meanwhile, this difference was greater in the MA+AL-treated rats compared to the rats treated with each of the drugs alone

(Figure 6C). Nevertheless, a significant decrease in the level of this parameter was observed 14 days after the cession of drugs (Figure 7).

A three-way ANOVA conducted on CAT activity showed no significant main effect of MA treatment, AL treatment and withdrawal period, and also their two- or three-way interactions (data not shown).

Discussion

The aim of the present study was to assess the effects of withdrawal from chronic MA and AL, either alone or in combination, on body weight, spatial learning and memory, as well as the status of oxidative stress in the hippocampus of adult male rats. Rats were gently handled

Figure 6: The effect of MA and/or Al treatments on oxidative stress markers. Panel A shows the effect of MA treatment on SOD activity. The level of SOD activity was significantly decreased in MA-treated rats compared with saline-treated rats (*p<0.0001). Panel B shows the effect of MA and/or Al treatments on the GPX activity. The GPX activity was significantly reduced in MA+AL-treated rats compared to saline-treated rats (*p<0.01). Panel C shows the effect of MA and/or AL treatments on the MAD level (*p<0.05). Both AL and MA increased the level of MAD in the hippocampus compared with saline-treated rats. However, in rats treated with MA and AL+MA, this difference was significantly greater than rats treated with AL alone. Data are means ± SEM.

Figure 7: The effect of withdrawal period on the MAD level. The MDA level was significantly reduced during the 2-week drugs withdrawal period (*p<0.05). Data are means ± SEM.

during weighing, injection and gavage, daily, which should eliminate stress effect on spatial learning and memory, as well as oxidative stress parameters.

Effect of MA and/or AL on Body Weight

Our results revealed an effect of chronic MA, but not AL and MA+AL, treatment on the loss of body weight in rats. The administration of MA resulted in decreased food intake and extreme weight loss, which is in agreement with previous studies [58,59]. Interestingly, the body weight loss continued even after the end of MA administration. It is well known that MA increases physical activity and suppresses appetite and then leads to anorexia and rapid weight loss extremely [60]. However, it should be noted that these effects are short term and body weight is regained after MA withdrawal [61]. Despite this, our results indicated that the body weight loss continued even after the end of MA administration. In other words, MA-induced weight reduction endured for at least two weeks after drug withdrawal. One potential explanation for this result is that the chronic MA-treatment may result in functional changes in either lateral or ventromedial nuclei of the hypothalamus [62]. However, some studies have suggested that the mechanism underlying the appetite suppression and anorexigenic effect of MA and other derivatives of amphetamine may be mediated by leptin, ghrelin, growth hormone (GH), and hypothalamic neuropeptide-Y (NP-Y) [63,64]. On the other hand, various researches have reported the mediation of both types of dopamine D1/D2 receptors and NPY in amphetamine-induced appetite suppression [65].

Effect of MA and/or AL on Spatial Learning and Memory and oxidative stress markers

Considerable evidence suggests that MA and AL co-abuse may have a greater impact on brain structures and functions than each treatment by itself [52]. In order to simulate the human patterns of MA abuse, gradually escalation doses of MA for 14-day, with a 14-day multi-binge period starting on day 15 were used in the present study [66].

It has been suggested that the toxic effects of MA on cognitive function depend largely on the pattern of abuse [32,33]. According to some investigations an escalating-dose multi-binge MA administration leads to tolerance to its cognitive impairing effects [38,39]. For instance, Clark, et al. [66] has reported that escalating-dose MA administration failed to impair recognition memory in rats. On the other hand, there are studies reporting impairments of cognitive function induced by escalating doses of MA exposure [67].

The present study revealed that an escalating-dose multi-binge pattern of MA administration resulted in marked impairment in spatial memory. During the acquisition phase, MA significantly increased the latency time and swim path length to find the hidden platform. Similarly, MA treatment decreased the time spent and the traveled distance in the target quadrant during the probe trial. Furthermore, MA-induced learning and memory deficits were observed two weeks after the last MA injection. Consistent with previous studies [16], these results indicate that chronic MA administration induces long-lasting spatial learning and memory impairment. One possible explanation for this result is that the tolerance induced by escalating doses of MA is not permanent. In other words, it persists for few days and then declines until it is completely eliminated. This hypothesis is in agreement with results of Danaceau, et al. [68]. They evaluated the persistence of tolerance to monoaminergic deficits conferred by escalating-dose methamphetamine treatment. Their results revealed that tolerance induced by escalating doses of methamphetamine

persists for one to two weeks but is completely eliminated by 31 days. However, the discrepancies between results of this and other studies can be related to the doses of MA administered, drug administration times in a day, duration of binge period (in which the animals were exposed to administrations of high doses of MA), memory paradigms used, as well as age and strain of animals used. In our study, in line with others [69-71], moderate-dose AL alone caused no evident impact on spatial learning and memory while MA and AL co-administration produced a profile of effects similar to, but more severe than, those caused by MA alone. In accordance with the present results, previous studies have shown synergistic disruption of cognitive function by co-administration of MA and AL in both animals and humans [72,73]. It is well documented that spatial memory is dependent on the integrity of the hippocampus [74], and damage to this region, thus impairs its role in spatial learning and memory [68]. MA can cause neuronal death [75], especially the loss of pyramidal neurons in the hippocampus [24]. In addition, some pyramidal neurons show loss of their dendrite complexity and dystrophic neuritis consistent with neurodegeneration in the frontal cortex and in both CA1 and CA3 regions of the hippocampus [24]. Also, Meth decrease hippocampal LTP [76,77]. Furthermore, MA abuse results in disruption of the Blood-Brain Barrier (BBB), particularly in the cortex and the hippocampus that persist even in abstinent MA users [78-80].

Although the exact mechanisms underlying the effects of MA on memory function are unclear, there is increasing evidence suggesting that oxidative stress may play an essential role in the MA-induced cognitive function disturbance [81]. The role of oxidative stress in MA-induced toxicity is further supported by the observations that administration of antioxidants can attenuate MA-induced cellular toxicity [80]. Therefore, the effects of the escalating doses of MA and/or AL combination on oxidative stress in the hippocampus were investigated in the present study. Oxidative stress is defined as a disturbance in the balance between the production of free radicals such as Reactive Oxygen Species (ROS) and antioxidant defenses [82,83]. In all cells, ROS are chemically very reactive molecules containing oxygen. They continuously generated as a by-product of the normal metabolism of oxygen and are involved in intracellular signaling and in ATP generation. ROS can be produced at multiple sites in a mammalian cell. Mitochondria are the energy machinery of the cell and the major sites for the production of ROS [84]. Increases in ROS can damage cells via oxidation of essential biomolecules such as lipids, DNA and proteins. ROS mainly attack the cellular membrane Lipids and cause lipid peroxidation [85,86]. Lipid peroxidation is a process resulting from damage to cellular membranes mediated by ROS that generate several relatively stable end products, including MDA that can be measured in plasma or tissues as markers of oxidative stress [87]. ROS normally are detoxified by antioxidant enzymes (i.e. SOD, catalase, and GPX). The brain, particularly the hippocampus, is highly sensitive to any imbalance in ROS due to high oxygen consumption, high iron and lipid contents, and low activity for antioxidant defenses [88,89]. It has been shown that ROS impair the neuronal networks necessary for memory function [84].

Our results revealed that the escalating-dose multi-binge MA administration decreased SOD activity and increased MDA level in the hippocampus tissue. The reduction in the antioxidant defense mechanisms increased the oxidative stress in the hippocampus and provided a reasonable explanation for the memory deficits accompanying MA exposure. These results confirm previous reports that chronic MA exposure leads to oxidative injury in the hippocampus [81]. In addition, our data showed that chronic AL

treatment significantly increased concentrations of MDA but failed to significantly affect any other marker of oxidative stress. In line with the present study, it has been reported that AL exposure causes an increase of MDA levels, without changing the SOD and GPX activities in the hippocampus [90,91]. Although according to some studies, increased oxidative stress in hippocampus due to ethanol treatment results in memory disorders [92], however, we detected no such correlation. A possible explanation could be that, spatial memory deficit may depend on the amount of damage to the hippocampus [74]. Although AL-induced oxidative stress can damage the hippocampus, but the amount of the damage was not enough to effect spatial memory performance.

Notably, while both drugs significantly induced oxidative stress in the hippocampus when given alone, co-administration of these drugs was accompanied by a higher oxidative stress. Indeed, MA+AL co-administration decreased both SOD and GPX activities and increased MDA level. This indicates higher production of ROS and antioxidant defenses reduction in the hippocampus and provided a reasonable explanation for sever neurotoxic effects of MA and AL combination.

In conclusion, this study demonstrates that escalating-dose; multi-binge pattern of MA and AL co-administration has synergistic effects on increased oxidative stress in the hippocampus, as well as spatial memory impairment.

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